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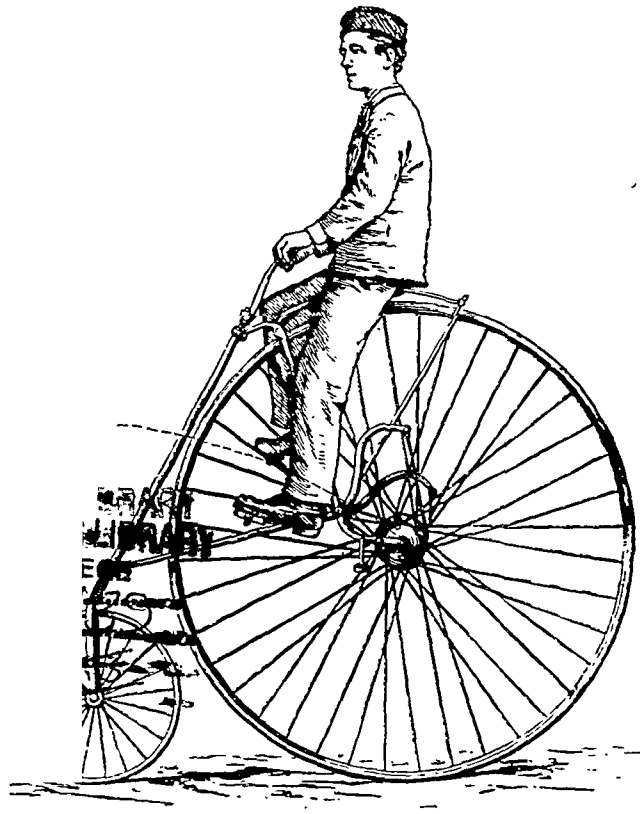

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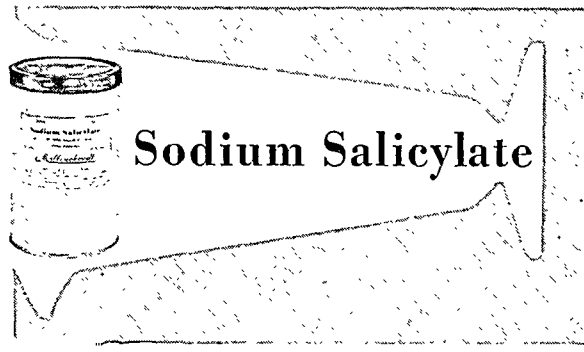

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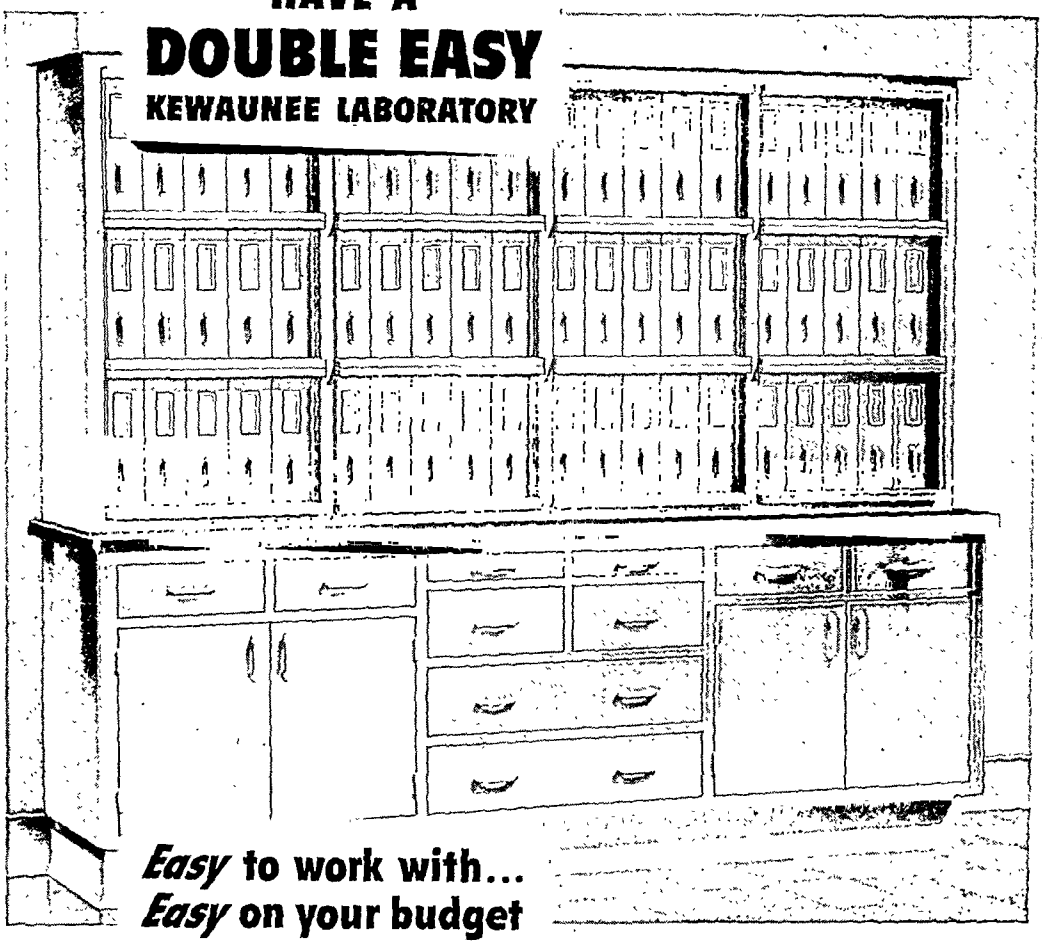

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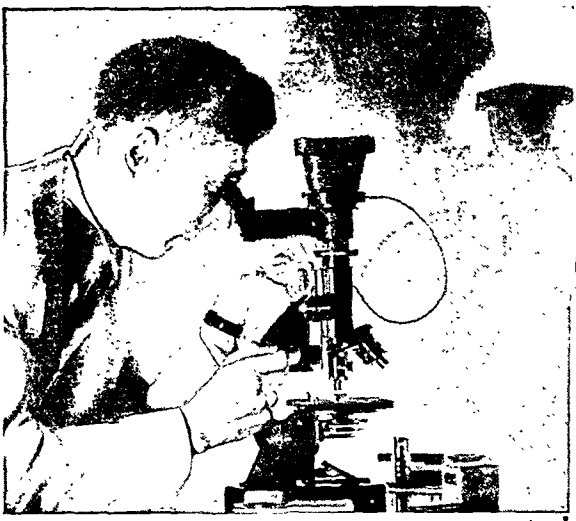

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VOLUME XXXVII | *FEBRUARY, 1948* | NUMBER 2 CONSECUTIVE NO. 4

Some Unsolved Problems in the Chemistry of the Nucleic Acids*†

By R. STUART TIPSON

The nucleic acids are of immense importance in the chemistry of life-processes since they are found in the nucleus and cytoplasm of every cell. A brief summary of those details of their chemical structure which are known with certainty is presented; those which are still unknown or based purely on analogy are pointed out, and some suggestions for further research are made.

SUITABLY isolated, the molecules of nucleic acids are normally of exceedingly high molecular weight. It appears that the macromolecules of any one kind of nucleic acid may vary in size according to their source. The size is certainly dependent on the treatment to which the material is subjected during its isolation (1); this may involve partial depolymerization, dephosphorylation, or other degradation. The matter of the actual size of these various macromolecules will not be discussed here, except to say that the average molecular weight of the macromolecule of one nucleic acid examined may be of the order of half a million to a million (2); of another (3), about 37,000; of another (4), about 16,000 to 23,000.

We still know amazingly little concerning the manner in which these macromolecules are built up but there seems to be no doubt that a macromolecule is composed of a large number of mononucleotides. One way, but not the only possible way, of interpreting some of the results obtained is as follows:

Nucleic Acid Macromolecule
↓ (depolymerization)
Fundamental Polynucleotide Unit
↓ (hydrolysis)
2 purine bases (1 mole each)
+2 pyrimidine bases (1 mole each)
+a sugar (4 moles)
+phosphoric acid (4 moles)

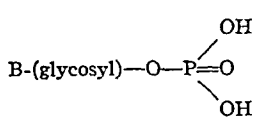
There are a number of reasons for believ-

* Received Aug. 5, 1947, from the Department of Research in Pure Chemistry, Mellon Institute, Pittsburgh 13, Pa.

† Based on a paper presented at the meeting of the Division of Biological Chemistry, American Chemical Society, held in Atlantic City, N. J., on April 15, 1947; revised since then in the light of certain recent discoveries. Parts of this discussion had already been published in *Advances in Carbohydrate Chemistry*, Academic Press, Inc., Vol. I, 1945, pp. 193–245.

ing that the hypothetical "fundamental polynucleotide unit" may exist, but this has not been proved beyond doubt. On the other hand, there appear to be, as yet, no reasons for believing that it does not exist. Claims that the fundamental unit is set free by enzymic (5) and chemical (6) depolymerization need substantiation. If the material is isolable it should be characterized carefully, because there is no proof that its constituent parts are always combined in the same way, in an invariant order. Furthermore, there appears to be no proof that any one macromolecule is an exact multiple of the supposed polynucleotide unit. It seems that some information on this point might be gained by taking various samples of a selected nucleic acid, each sample representing one molecular size (or as near as possible to it), and hydrolyzing each in order to see if the proportions of hydrolysis products are the *same* for each sample. Finally, if there is such a polynucleotide unit which, by repeated self-combination, yields the macromolecule, there is as yet no assurance that the inter-polynucleotide union is uniform throughout the macromolecule.

However, as regards the simple organic chemistry of the nucleic acids, these questions are of no immediate importance. We shall assume, as a working hypothesis, that any one macromolecule is a polymer of a fundamental polynucleotide molecule which may be defined chemically if we know (*a*) the number of its constituent mononucleotides, (*b*) the precise details of structure of each mononucleotide, and (*c*) the way and sequence in which the mononucleotides are united to give the fundamental polynucleotide. The mononucleotides are of two types, phosphoglycosyl-purines and phosphoglycosyl-pyrimidines, respectively:



where B = a purine or pyrimidine residue. Complete structural knowledge of a mononucleotide therefore involves determination of (*a*) the nature of the base; (*b*) the nature of the sugar; (*c*) the ring structure of the sugar residue; (*d*) the stereochemical disposition (α- or β-) of the sugar-base link; (*e*) the position of union of the sugar to the base; and (*f*) the position of the phosphoryl group on the sugar chain.

It is generally accepted that there are two main types of nucleic acid, similarly constituted but differing in certain components. Since the striking difference in the chemical and physical properties of these two acids is occasioned by the properties of their sugar components, they are now known as pentose-nucleic acid and desoxypentose-nucleic acid, respectively. There have been indications from time to time that other nucleic acids exist, but only these two types have been adequately characterized from the point of view of the organic chemist.

PENTOSENUCLEIC ACID

Let us consider the pentose-nucleic acid isolable from yeast. The bases were identified long ago as the purines adenine and guanine, and the pyrimidines uracil and cytosine. The four corresponding mononucleotides are adenylic acid, guanylic acid, uridylic acid, and cytidylic acid. We now know all the details of structure of the nucleotides. In the phosphopentosylpurines, the sugar is *D*-ribose (7), the configuration is β- (8), the sugar is attached at position 9′ of the purine (9), the sugar ring-structure is furanose (10), and the phospho group is situated at position 3 of the sugar residue (11). In 1946, Davoll, Lythgoe, and Todd (8) proved that adenosine has the β-configuration; since it is hydrolyzed by the same enzyme, guanosine probably has the same configuration.

Hence the pentosylpurine nucleotides are 3-phospho-9′-β-*D*-ribofuranosylpurines. They may be depicted as shown in Fig. 1.

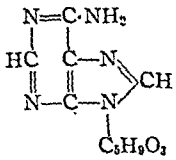
In the phosphopentosylpyrimidines, the sugar is *D*-ribose (12), the configuration is β- (8), the sugar is attached at position 3′ of the pyrimidine (13), the sugar ring-structure is furanose (14), and the phospho group is situated at position 3 of the sugar residue (15).

Hence the pentosylpyrimidine nucleotides are 3-phospho - 3′ - β - *D* - ribofuranosylpyrimidines. They have the formulas illustrated in Fig. 2.

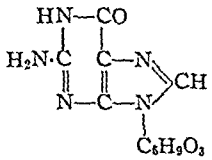
DESOXYPENTOSENUCLEIC ACID

The nitrogenous bases of desoxypentosenucleic acid are adenine, guanine, cytosine, and thymine (5-methyluracil). Levene discovered (16) that the sugar of the purine nucleosides is 2-desoxy-*D*-ribose. By ultraviolet absorption spectra studies, it has been shown to be attached at position 9 of the purines

(17), but its ring-structure has not been proved, and it is unknown whether these nucleosides are of α- or β-stereochemical configuration.

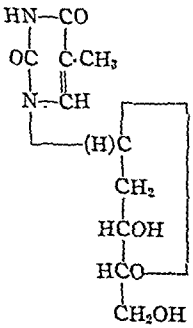


Desoxyribosyl-adenine

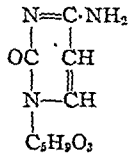


Desoxyribosyl-guanine

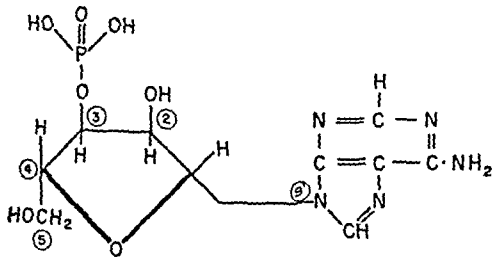
To date, there appears to be no proof at all that the sugar of the two desoxypentosyl pyrimidines is 2-desoxy-*D*-ribose, although there are indications that it is a 2-desoxypentose. From theoretical considerations of a chemical nature, it is almost certainly attached at position 3′ of the pyrimidine bases, but there is no positive proof of this. Evidence has been adduced indicating that the ring-structure of the sugar in thymidine is furanose (18), but that of the sugar in the cytosine nucleoside is unknown. It is not known whether these nucleosides are of α- or β-stereochemical configuration.



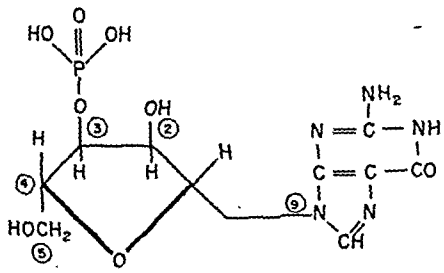
Thymidine



Desoxypentosyl-cytosine

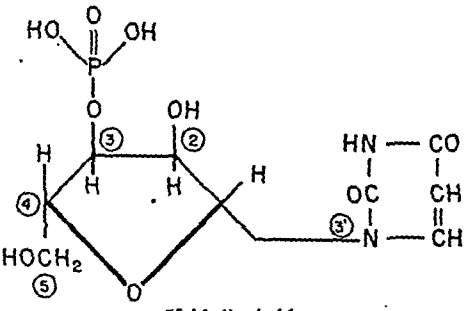


Adenylic Acid

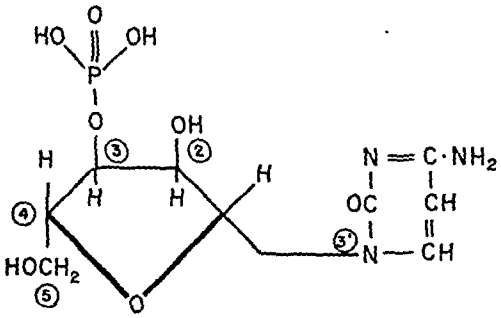


Guanylic Acid

Figure 1



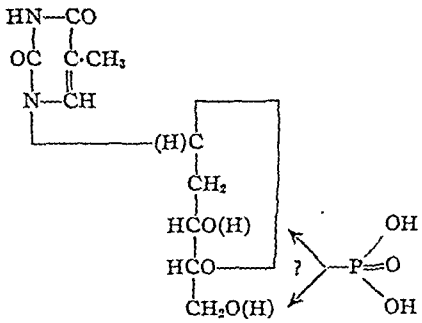
Uridylic Acid



Cytidylic Acid

Figure 2

Turning now to the corresponding nucleotides, the position of attachment of the phosphoryl group on the sugar is unknown. It cannot be at position 2 and if, as appears likely, the desoxypentose residue is furanose, it cannot be at position 4. Hence, it is probably either at position 3 or position 5. These

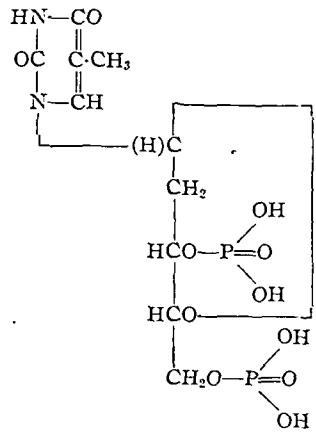


Monophospho-thymidine

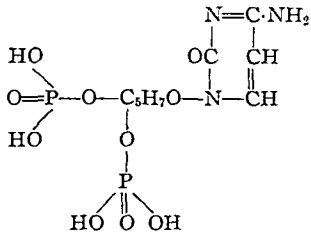
two pyrimidine nucleotides should be hydrogenated and then hydrolyzed with dilute mineral acid, and the resulting monophosphodesoxypentose studied. It will probably prove to be 3-phospho-2-desoxy-*D*-ribose, but this is still a matter of conjecture.

Now, in 1912, Levene and Jacobs (19) discovered that, on hydrolysis of the desoxypentosenucleic acid by means of boiling 2% sulfuric acid during two hours, diphosphoric esters of thymidine (20) and of the cytosine nucleoside (21) may be isolated. On catalytic hydrogenation, diphosphothymidine gave diphosphodihydrothymidine, a substance readily hydrolyzed by mineral acid to dihydrothymine and a reducing diphospho-sugar. Since thymidine is a

furanose derivative, the phosphoryl groups must be



Diphosphothymidine



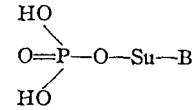
Diphospho-desoxypentosylcytosine

situated at positions 3 and 5 of the sugar. The diphospho-sugar should receive further study, since it offers the chance of identifying the sugar of these pyrimidine nucleotides; there is every reason to believe that it is 2-desoxy-*D*-ribose, but this has not yet been proved.

Having pointed out those features of structure of the mononucleotides which are still unknown, we may pass to the fundamental polynucleotide unit. Assuming that there *is* such a unit, the first point to be settled is the number of mononucleotides comprising it. Let us first consider *ribose-nucleic acid.*

Complete hydrolysis of the nucleic acid gives the four nitrogenous bases in equimolecular proportions (22). Mild alkaline hydrolysis of the nucleic acid gives directly an equimolecular mixture (23) of the four mononucleotides. Hence it appears reasonable to believe that the fundamental unit is a *tetra*nucleotide composed of adenylic acid, guanylic acid, uridylic acid, and cytidylic acid. It has been supposed that every such unit is identical with every other and that this unit consists of one molecule of each of the four nucleotides, always combined in the same relative order (sequence) and in the same way. Real proof of these speculations is still not at hand, but the available facts tend to favor the idea of a poly-tetranucleotide structure for yeast ribonucleic acid and for some desoxyribonucleic acids.

The next question which arises is, how, and in what order, are these four mononucleotides united. Combination between any two individual mononucleotides having the general formula



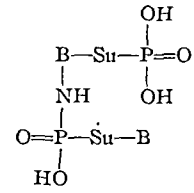
where Su = sugar residue, and B = purine or pyrimidine base, might be visualized as taking place in at least four different ways:

(*a*) through a phosphoamide link, the phosphoryl group of one nucleotide uniting with the amino group of the other (adenylic, guanylic, or cytidylic acid);

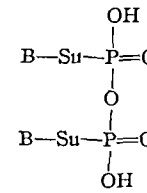
(*b*) through a pyrophosphate link, one phosphoryl group being combined with that of the other nucleotide;

(*c*) through an ether link, from a hydroxyl group of one ribose residue to one of the other ribose residue; or

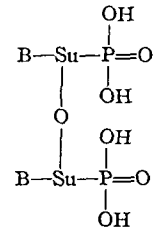
(*d*) through an ester link, the phosphoryl group of one nucleotide uniting with a hydroxyl group of the ribose residue of the other nucleotide, or with a lactim hydroxyl of one of the nitrogenous bases.



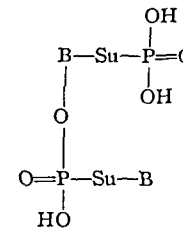
Phosphoamide



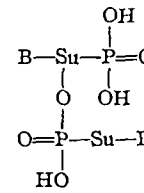
Pyrophosphate



Ether



Lactim-ester

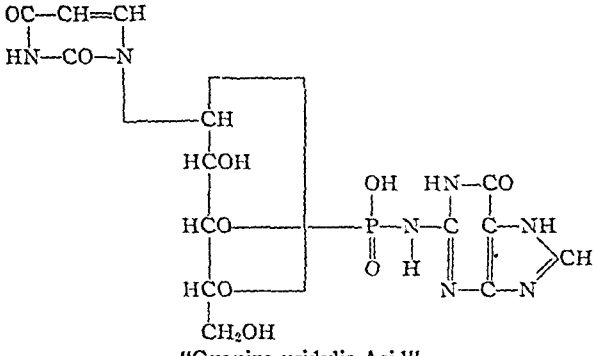


Diester

At one time or another, every one of these modes of union (and various combinations of them) has been advocated.

Since the three primary amino groups in ribosenucleic acid can be determined (24) by van Slyke's method, the possibility of phospho-amide linkages may be dismissed. In 1936, Bredereck (25) hy-

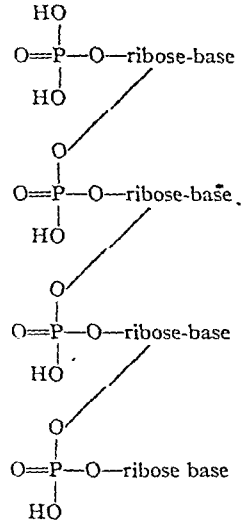
drolyzed some Boehringer ribosenucleic acid and isolated a very small amount of a material which he thought was "guanine-uridylic" acid of the formula



"Guanine-uridylic Acid"

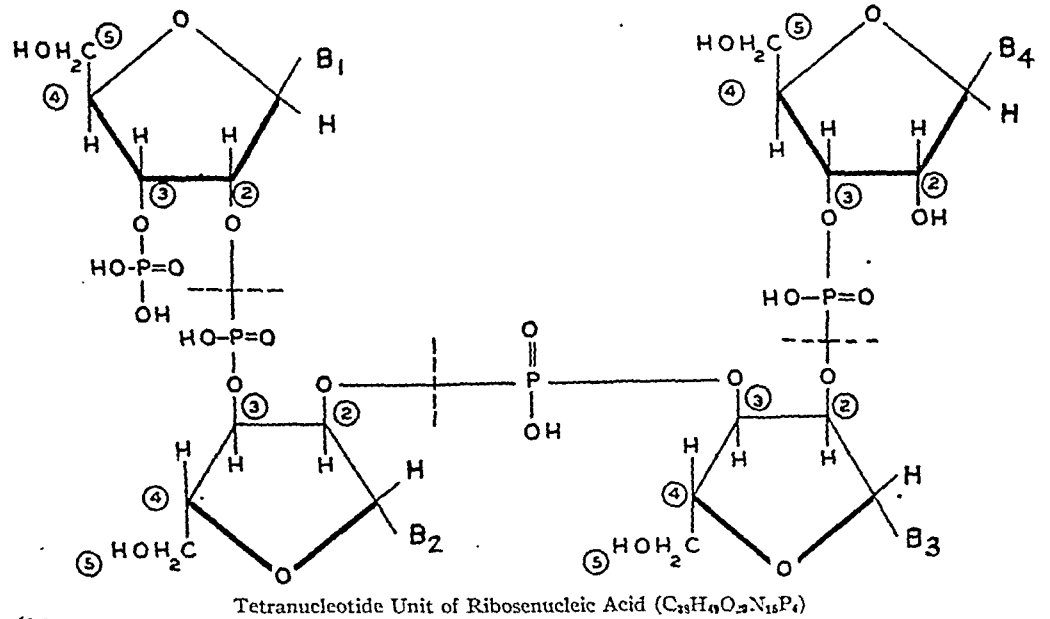
but it is now believed that this compound is an artifact, formed during the hydrolysis.

For various reasons, formulas involving pyrophosphate, ether, or lactim-ester linkages have been discarded. The formula at the right, proposed (26) by Levene in 1921, still seems the most likely. It depicts the fundamental polynucleotide as a tetranucleotide in which there are three di-ester phosphoric linkages between the individual mononucleotide residues. It was then suggested (27) that the tetranucleotide unit may be more precisely depicted as shown in Fig. 3. In this formula, each of the three linking phosphoryl groups is shared by position 2 of one ribose residue and position 3 of the adjacent ribose residue, so that, on hydrolysis, the four 3-phosphonucleotides result. It will be noted that the four primary sugar hydroxyl groups and one secondary hydroxyl are unsubstituted. Hence, it is suggested that some light might be thrown on this problem by appropriate alkylation or acylation of the fundamental tetranucleotide with subsequent hydrolysis to one di-derivative of ribose and three mono-alkyl or monoacyl derivatives.



Levene's Phospho-diester Formula for Ribosenucleic Acid Tetranucleotide

Many attempts have been made to decide the kind and position of the phosphoryl linkages by study of the number of phosphoric acid dissociations. Perhaps the first problem is to isolate pure tetranucleotide for study; then various homogeneous polymers of known molecular size should be examined.



Tetranucleotide Unit of Ribosenucleic Acid ($C_{38}H_{49}O_{32}N_{15}P_4$)
(Adapted from Tipson, R. S., "Advances in Carbohydrate Chemistry," Academic Press, Inc., Vol. I, 1945, p. 222.)

Figure 3

The tetranucleotide of Levene's formula should display one secondary and four primary phosphoric dissociations; and if a number of such units are joined in a chain through secondary phosphoric acid groups, there will only be one singly-bound phosphoric group present (terminally). This is depicted in the following abbreviated formula, in which all hydroxyl groups shown are attached to phosphorus:

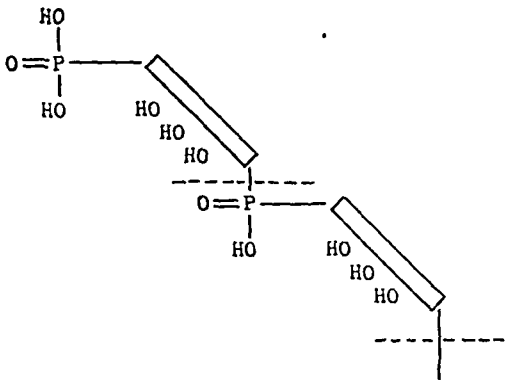


Figure 4

If the chain is long, it will be difficult to estimate the presence of this single group. Provided that the secondary phosphoric group of one tetranucleotide unit does not engage a phospho group of the adjacent tetranucleotide unit, the longer the chain, the closer will the number of acid dissociations approach four. According to Allen and Eiler (28), the polymerized acid does have four primary phosphoric dissociations per tetranucleotide unit, and, on treatment with ribonuclease (then believed to depolymerize the polymer to the tetranucleotide), one secondary phosphoric dissociation per unit appears. Levene (29) described a sample of ribosenucleic acid having 4.5 acid dissociations per tetranucleotide unit; it may have been partially depolymerized. Cohen (30) has shown that both ribosenucleic acid and desoxyribosenucleic acid may possess either four or five acidic groups per tetranucleotide unit, depending on the previous treatment. A careful correlation of molecular size with number of phosphoric dissociations and with hydrolysis products should be attempted.

If the secondary phosphoric group of one tetranucleotide engages a primary phosphoric group of the adjacent tetranucleotide unit, and this occurs repeatedly, Levene's formula would give a polymer having approximately three primary phosphoric dissociations. Levene (29) described such a sample; he considered that it probably possessed anhydride linkages. If intertetranucleotide linkage occurs (Fig. 5), through primary groups, the polymer will possess (approximately) one secondary and three primary dissociations per tetranucleotide unit. However, as shown in Fig. 6, it is perfectly possible that both types of intertetranucleotide linkage are present, namely, through secondary and through primary groups. There may even be cross-linkages from one chain to another. Alternatively, the polymer might consist of superimposed layers of a cyclic tetranucleotide. The possibility of hydrogen bonding should not be overlooked.

More recently, Gulland (31) has found, by electrometric titration, that yeast ribosenucleic acid has four acid dissociations per tetranucleotide unit, three of which are primary dissociations and one a secondary dissociation of phosphoric acid. Diminution of the molecular weight of the polytetranucleotide by mild alkaline hydrolysis gave results suggesting that a further secondary dissociation of phosphoric acid is set free, i.e., the fundamental tetranucleotide should be pentabasic, with three primary and two secondary dissociations. According to Gulland's results, possible formulas for the tetranucleotide (which polymerizes at A or B) are as shown in the formulas on the opposite page. However, if polymerization involves groupings at A and B, or from A or B of one tetranucleotide unit to a different phospho group of another unit, these formulas would give the wrong number of dissociations for the polymer. In polymers of such a tetranucleotide, it would be possible to split off as

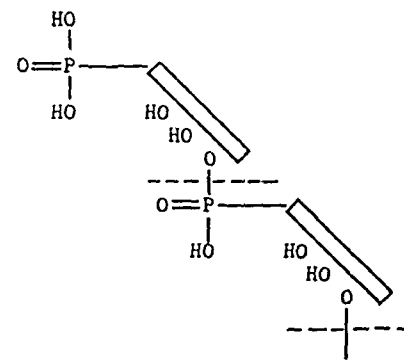


Figure 5

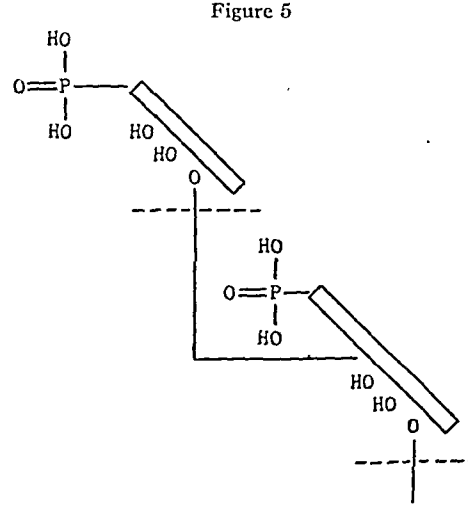


Figure 6

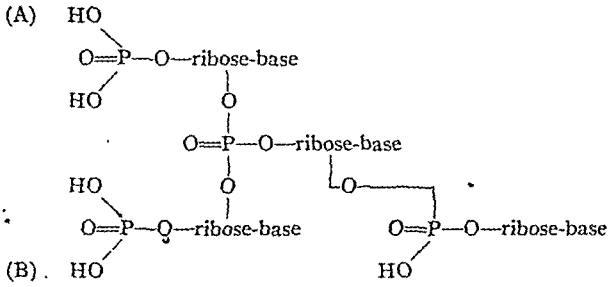
much as 25% of the total phosphorus without changing the main polynucleotide structure.

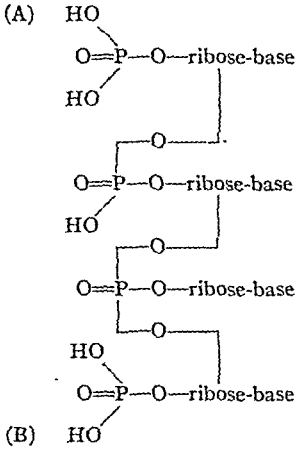
If some nucleotides were present as side chains, these might be removed (e.g., by ribonuclease) leaving a molecule still large enough to resemble the original acid in precipitability and in failure to dialyze. Indeed, Doring's work (32) on the action of ribonuclease on the polymer suggests that the "acid must contain at least two different types of linkages, one of which is labile and one of which is resistant" to the action of ribonuclease. Chemical hydrolysis (31) of the polymer apparently also causes fission of a link involving a secondary acidic group of a phosphoryl radical. It would be of interest to find out whether the products are identical, and whether the same phospho linkings are split in each type of degradation or depolymerization.

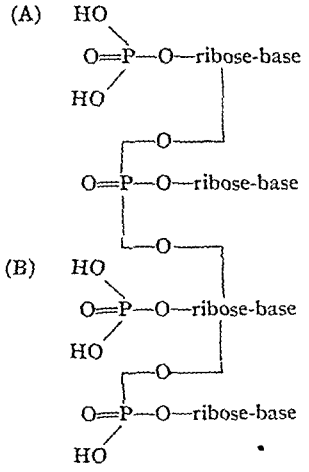
Finally, *there is the question of the order in which* the four nucleotides are united. Bredereck (33) has shown that mild hydrolysis of ribosenucleic acid with aqueous pyridine at 100° gives guanylic acid (G) plus a tri-nucleotide of adenylic acid (A), cytidylic acid (C), and uridylic acid (U). On further hydrolysis, A is split off. Bolomey and Allen (34) found that a certain nonspecific phosphatase preparation hydrolyzes ribosenucleic acid so that guanosine is, at first, liberated faster than adenosine. This suggests that guanylic acid is the first mononucleotide liberated, and adenylic acid the second. Hence, if de-

phosphorylation is not preceded by disruption to the *four* mononucleotides, guanylic acid is at one end of the chain, and adenylic acid is either next to it or at the other end. Possible formulas for the union of these are therefore:

G—U—C—A,
G—A—U—C,
G—A—C—U,
and G—C—U—A.







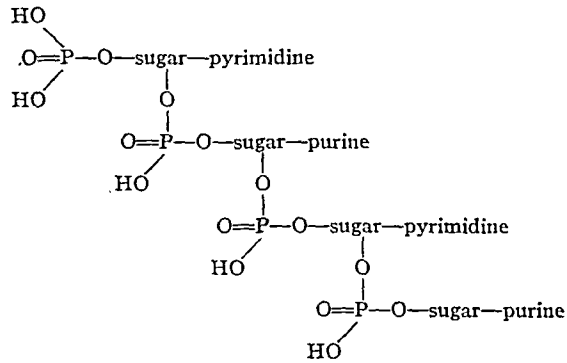
It would be interesting to arrest the hydrolysis when liberation of adenosine and guanosine approaches the maximum, and ascertain whether the pyrimidine nucleotides are present as a dinucleotide or as the two mononucleotides.

Turning now to the fundamental polynucleotide unit of *desoxyribosenucleic acid*, if such there be, the first point to be settled is the number and kind of mononucleotides comprising it.

Levene (35) showed that the two purine and the two pyrimidine bases occur in the acid in equimolecular proportions. Enzymic hydrolysis (36) gives all four nucleotides. Hence it appears reasonable to believe that the fundamental unit is a *tetra*nucleotide.

Levene (29, 37) and Bredereck (38) find that the acid, presumably depolymerized, has one secondary

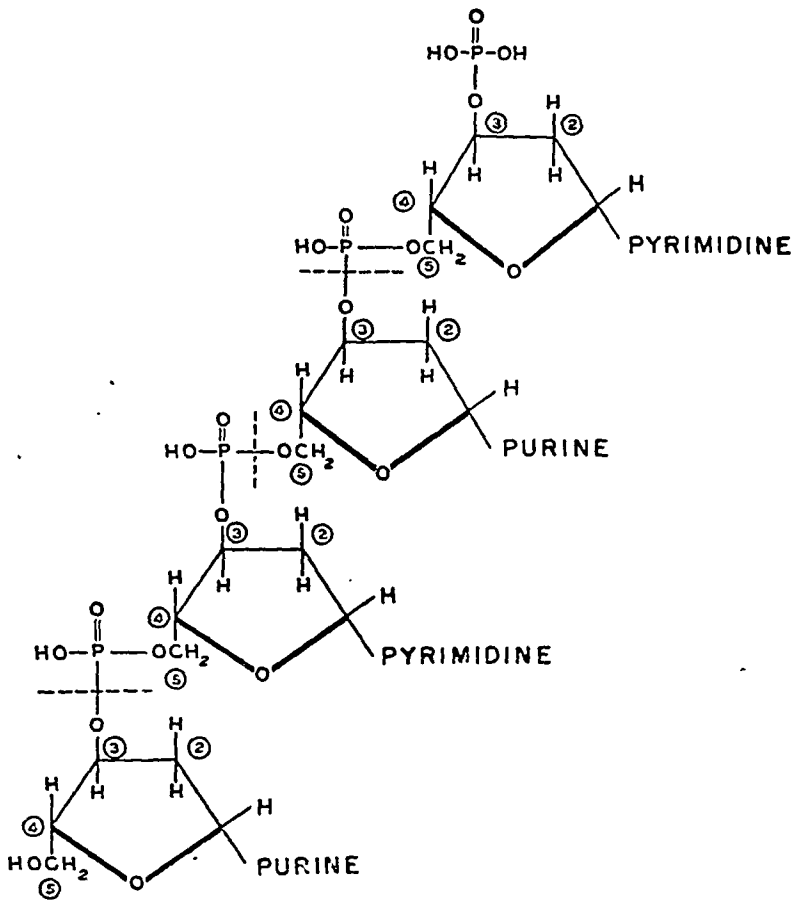
and four primary phosphoric dissociations, and since on suitable hydrolysis (19) it gives two diphosphodesoxypentosylpyrimidines, it has alternating purine and pyrimidine residues as follows:



Accepting the furanose structure for thymidine, and assuming it for the other three nucleosides, the phospho groups must join positions 3 to 5, as shown (27) in Figure 7 below.

On deamination it retains its tetranucleotide structure and pentabasicity (33), and hence contains no phosphoamide links. Evidence has been adduced recently (39*a*) that, in the polymer, hydrogen bonding may occur between purine and pyrimidine hydroxyl groups and some of the amino groups.

The polymerized acid is said (28, 39, 40, 41) to possess four acidic groups per tetranucleotide unit,



Tetranucleotide Unit of Desoxyribosenucleic Acid ($C_{39}H_{51}O_{25}N_{15}P_4$)
(Adapted from Tipson, R. S., "Advances in Carbohydrate Chemistry," Academic Press, Inc., Vol. I, 1945, p. 243.)

Figure 7

but a correlation between average basicity and average molecular weight does not appear to have been made. Cohen's studies (30) show that the acid may contain either four or five acidic groups per tetranucleotide unit, depending on the previous treatment. Depolymerization, either chemically (6) or with a depolymerase (42), appears to give a pentabasic tetranucleotide.

Finally, there is the question of the order in which the four nucleotides are united. It has previously been mentioned that the purines and pyrimidines alternate. By dephosphorylation (42, 43) of the supposed simple tetranucleotide, the nucleosides liberated first were those of adenine and thymine (T). This suggests that these are at the end or ends of the tetranucleotide chain, for example, thus:

A—C—G—T, and A—T—G—C.

Formulas A — T — C — G and A — G — C — T do not have alternating purines and pyrimidines and so are eliminated. Further study along these lines might permit us to decide which represents the true structure.

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Use of *Bacillus Subtilis* in the Three-Hour Cylinder-Plate Assay for Penicillin*

By ROBERTSON PRATT, FRANK M. GOYAN, JEAN DUFRENOY, and LOUIS A. STRAIT with the Laboratory Assistance of TOINIE JUNTUNEN and VIRGINIA LAMB†

The method of penicillin assay involving physical development of seeded plates has been extended to include plates seeded with *Bacillus subtilis*. The optimum conditions for tests with this organism are described. Advantages of using *B. subtilis* instead of *S. aureus* in this procedure are that assays can be completed more rapidly with the former organism, and that, with optimum conditions, the demarcation between areas of inhibition and of noninhibition is sharper. This rapid assay method provides reliable estimates of total penicillin activity in mixtures of different penicillins but is not suitable for performing "differential" assays.

SEVERAL procedures for assaying penicillin that retain the advantages inherent in the cylinder-plate method, but that reduce the time required for testing a solution of unknown concentration from about sixteen hours to three hours or less have been reported (1, 2). Of the several methods described, the one in which the test plates are rendered analogous to photographic emulsions has proved most useful for routine assays. This technique, details of which have been published (1), was developed using *Staphylococcus aureus* as the test organism.

The technique is not limited to this organism, however, but may be applied satisfactorily, *mutatis mutandis*, to plates seeded with other bacteria. Since a rough form of *Bacillus subtilis* (NRRL strain No. B-558) is commonly employed in performing assays of mixtures of different penicillins, it seemed of interest to determine the optimum conditions for physical development of plates seeded with this organism and to ascertain whether satisfactory subtilis/aureus ratios can be secured by the rapid method. This ratio varies for different penicillins and has been used as an indication of the proportions of different penicillins present in mixtures (3, 4).

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† Acknowledgment is made to the Cutter Laboratories, Berkeley, Calif., for a generous grant that made execution of this work possible.

EXPERIMENTS AND RESULTS

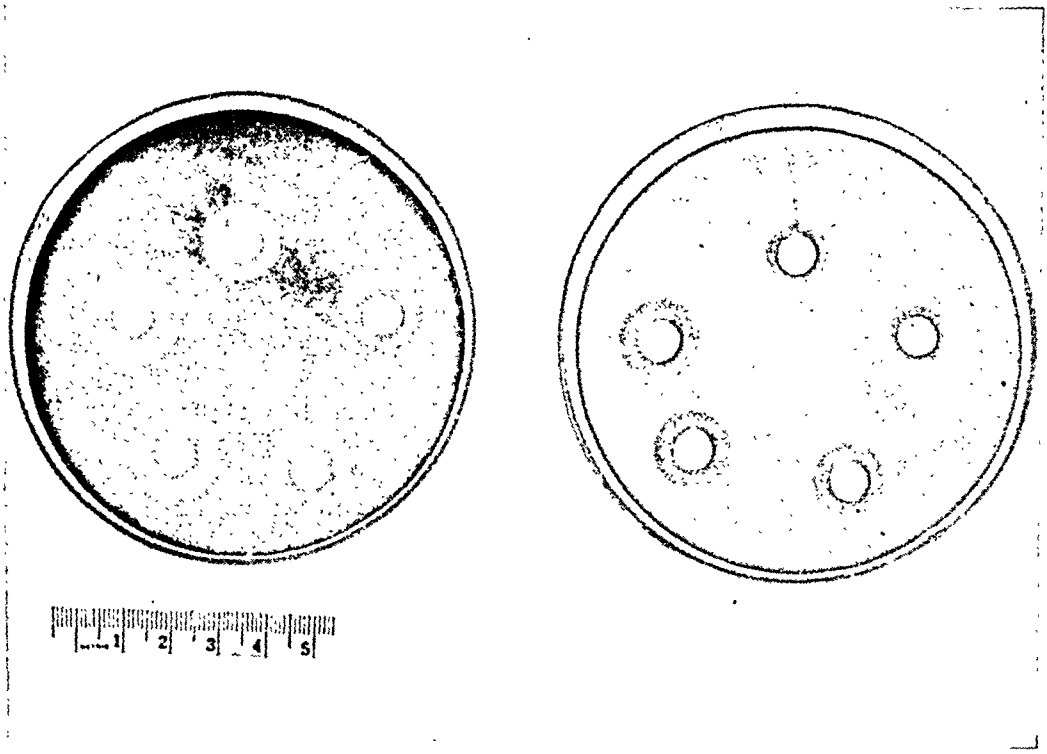
Physical Development of Plates Seeded with B. subtilis.—In general, the procedures to be used with *B. subtilis* are similar to those for *S. aureus*. They differ principally in the duration of the periods of incubation and of subsequent development. The incubation times for plates seeded with *B. subtilis* should be shorter, and should be more accurately controlled because duration of incubation is more critical with this organism than with *S. aureus*. It is important with *B. subtilis* that both primary and secondary incubation periods be relatively short and of approximately equal duration. When the ratio, primary incubation period/secondary incubation period, is markedly greater than 1.0, the plates are characterized by a uniform coarseness over the entire surface, and by small zones of inhibition which are of approximately equal diameter for all concentrations of penicillin from 0.5 to 8 units/ml When the ratio is less than 0.8, development of the entire plate is very weak, and extremely faint zones are obtained. The zones, however, show the expected correlation between diameter and concentration of penicillin.

This is in sharp contrast to *S. aureus* for which the duration of the primary and secondary incubation periods is not so critical.

The concentration of $AgNO_3$ solution that is used is less critical for assays involving *B. subtilis* than for those with *S. aureus*. In general, for a given set of conditions of temperature, concentration of silver nitrate solution, and of exposure to silver nitrate and to light, a longer period of development is required for plates seeded with *B. subtilis* The optimum conditions for developing plates seeded with the two different organisms are summarized in Table I.

The contrast between areas of inhibition and of noninhibition on plates seeded with *S. aureus* and subsequently subjected to a proper regimen of "physical development" is very pronounced, but the contrast between similar areas on plates seeded with *B. subtilis* is even more marked. Photographs of typical assay plates seeded with *B. subtilis* and with *S. aureus* and processed by physical development are reproduced in Fig. 1. Some definition has been lost in the processes of photography and reproduction.

Attention should be directed also to the different pattern that physical development reveals on assay



1A 1B

Fig. 1.—Reproduction of photographs of penicillin assay plates processed by impregnation with silver followed by exposure to light and subsequent physical development. Zones for solutions containing 1, 0.5, 2, 4, and 8 units/ml., respectively, are arranged in clockwise order beginning with those at the top. 1A (left), plate seeded with *S. aureus;* primary and secondary incubation periods each 3 hours. 1B (right), plate seeded with *B. subtilis;* primary and secondary incubation periods 2 hours and 2.5 hours, respectively.

TABLE I.—COMPARISON OF PHYSICAL DEVELOPMENT OF AGAR PLATES SEEDED WITH *S. aureus* AND WITH *B. subtilis* FOR ASSAY OF PENICILLIN

Condition	*S. aureus*	*B. subtilis*
$AgNO_3$		
Optimum concentration	0.1%	0.1–0.12%
Useful range of concentration	0.1–0.12%	0.1–0.16%
Primary incubation		
Optimum duration	3 hr.	2 hr.
Useful range	2.5–4 hr.	2–3 hr.
Secondary incubation with penicillin		
Optimum duration	3 hr.	2.5 hr.
Useful range	2.5–4 hr.	2–3 hr.
Time of development (at 22–25°)	7–10 min.	10–15 min.
Linear range of calibration curve (Log–Log)	1–8 units/ml.	1–8 units/ml.
Useful range of calibration curve	0.5–8 units/ml.	0.5–8 units/ml.

plates seeded with *B. subtilis* as compared with plates seeded with *S. aureus*. This can be seen in Fig. 1.

When primary and secondary incubation periods that give clearest development are chosen the calibration curves that are obtained by plotting log diameter of inhibition zone against log concentration of penicillin have approximately the same slope. The figures in Table II show average diame-

TABLE II

Diameter of Zone	Units of Penicillin/Ml. 0.5 Mm.	1.0 Mm.	2.0 Mm.	4.0 Mm.	8.0 Mm.
Observed	12.9	15.75	16.60	18.10	19.00
Calculated[a]	...[b]	15.73	16.76	17.89	19.05

[a] Statistically calculated value based on an assumed linear $\log_b$ diameter/log concentration function.
[b] No calculated value given for this concentration, since this range is slightly off the linear curve.

ters of zones of inhibition on plates seeded with *B. subtilis* and subjected to two hours of primary incubation and two and one-half hours of secondary incubation before physical development.

"Differential" Assays Employing B. subtilis and S. aureus as Test Organisms.—To ascertain whether satisfactory "differential" assays to determine the relative amounts of different penicillins in mixtures (3, 4) could be executed by the rapid cylinder-plate method employing physical development, different sets of plates were seeded with *S. aureus* and with *B. subtilis*, respectively, and were preincubated for two and one-half hours at 37°. Following addition of "unknown" solutions of penicillin, the plates were incubated again for two and one-half hours. The "unknown" solutions all contained one unit per ml., but each was composed of known different proportions of penicillins G and X. A solution containing one unit of penicillin G per ml. was used as a standard.

For comparison, differential assays using the same species of organisms and the same mixtures of penicillins were run by the standard sixteen-hour procedure. A complete set of "unknowns" and a standard were run on each plate. In general, the differential assays by the standard method were satisfactory and yielded results that conformed to expectations based on published values for subtilis/aureus ratios determined by this method (3). The differential values determined by the short method involving physical development varied in a random manner, however, and indicated that this technique is not adaptable to the performance of such assays. This result was not unexpected, since the inhibition zones that develop in the cylinder-plate assays represent the net visible expression of opposing factors, *viz.*, physical factors represented by diffusion, concentration gradient, etc., and biological factors represented by the growth of the organism. In the short methods, the biological factor (growth) is reduced to a minimum and the effect of the physical factors is relatively accentuated. Since relatively little difference would be anticipated in the rates of diffusion of penicillins G and X, it is reasonable that such a method should fail to reveal significant or consistent differences in the differential assays. On the other hand, the standard method tends to accentuate the importance of the growth factor; hence it reflects the different sensitivities of the two organisms to the two kinds of penicillin.

SUMMARY

The method of penicillin assay involving physical development of seeded plates has been extended to include plates seeded with *B. subtilis*. The optimum conditions for tests with this organism are described.

Shorter incubation periods may be employed with *B. subtilis* than with *S. aureus*. It is important that the primary and secondary incubation periods be of approximately equal duration when *B. subtilis* is used. The absolute and relative durations of the incubation periods are more critical for this organism.

The concentration of the $AgNO_3$ solution is less critical for development of plates seeded with *B. subtilis* than for plates seeded with *S. aureus*. However, plates seeded with the former require a longer period of exposure to the developer solution following treatment with $AgNO_3$ and exposure to light.

The rapid physical development method, as it is now employed, is not applicable to the determination of subtilis/aureus ratios that give a reliable indication of the proportions of penicillins X and G in mixtures of the two. The reasons for this are pointed out.

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Inactivation of the Germicidal Action of Quaternary Ammonium Compounds*

By C. A. LAWRENCE

The need for means of inactivating the antibacterial activity of quaternary ammonium germicides is summarized. Anionic detergents and soaps fail to inactivate surface-active cationic detergents. Suramin sodium is the most satisfactory inactivator of any in a group of sulfonates and sulfonic acids tested for this purpose.

It is of interest to note that following the introduction of effective chemotherapeutic or germicidal agents for the destruction of microorganisms, reports appear early in the literature on methods for the inactivation of the antibacterial compounds. These studies involve not only an attempt to explain the mechanism of action of the new derivatives, but also a means to determine the presence or numbers of living organisms that may remain dormant (inhibited) in dry powders or solutions of these products.

The present accepted sterility tests for liquids and solids require a medium containing thioglycollate (1, 2). The latter compound is used not only for the detection of aerobic and anaerobic microorganisms in a single procedure, but also for its ability to neutralize the bacteriostatic effect of mercurials that are added to many pharmaceuticals.

Additional examples of methods that are used for inactivating the antibacterial properties of various compounds may be found in the following combinations: sodium thiosulfate *versus* chlorine (3, 4); *p*-aminobenzoic acid *versus* sulfanilamide (5, 6); penicillinase *versus* penicillin (7, 8, 9); ferric chloride *versus* phenol (10); and glucose *versus* streptomycin (11).

With the increasing popularity that the quaternary ammonium germicides are receiving in the fields of medicinal and general disinfection, the need for a suitable means for inactivating the high germicidal activity of this group of compounds becomes apparent. In the inactivation of these germicides, as well as the antibacterial agents mentioned before, several important factors must be taken under consideration in selecting the most desirable means for accomplishing this. The neutralizing substance must, as completely as possible, inactivate the bactericidal and bacteriostatic properties of the compound and maintain this state over a prolonged period of time. The inactivating agent should, furthermore, have no antibacterial action itself and little, if any, growth-promoting properties upon microorganisms.

The quaternary ammonium germicides belong to a group of compounds known as "surface-active" agents or detergents. These may be classified as non-ionic, or ionic. The latter may be subdivided as (*a*) cationic, in which the lipophilic (oil-attracting) group becomes the cation (+); (*b*) anionic, in which the lipophilic group becomes the anion (−). The surface-active quaternary ammonium germicides ionize in water as cationic compounds in which the long chain part of the molecule is positively charged.

Cationic and anionic detergents will neutralize and often precipitate each other. Domagk (12), who called attention to the high antiseptic properties of the quaternary ammonium germicides, was first to indicate the neutralizing action of an anionic detergent (soap) upon a cationic detergent. Baker, Harrison, and Miller (13) noted the inhibiting action of the anionic compounds, decyl sulfate and sodium taurocholate, upon Benzalkonium Chloride (U. S. P. XIII). Of particular interest were the observations of Valko and DuBois (14) who noted that bacteria which are apparently "killed" by a cationic quaternary ammonium germicide may be revived by the addition of a high molecular anion, provided that application of the latter occurs within a time period of ten to thirty minutes.

* Received Oct. 3, 1947, from Winthrop Chemical Company, Inc., Rensselaer, N. Y.

TABLE I.—EFFECTS OF SEVERAL COMPOUNDS UPON ANTIBACTERIAL ACTION OF BENZALKONIUM CHLORIDE (1:11,000) AGAINST *S. aureus*—COLONIES PER 4-MM. LOOP

Test Agent	1 KCNS 15 Min. D[a]	1 KCNS 15 Min. C	1 KCNS 30 Min. D	1 KCNS 30 Min. C	2 $K_3Fe(CN)_6$ 15 Min. D	2 $K_3Fe(CN)_6$ 15 Min. C	2 $K_3Fe(CN)_6$ 30 Min. D	2 $K_3Fe(CN)_6$ 30 Min. C	3 Duponol C 15 Min. D	3 Duponol C 15 Min. C	3 Duponol C 30 Min. D	3 Duponol C 30 Min. C	4 Santomerse T. 15 Min. D	4 Santomerse T. 15 Min. C	4 Santomerse T. 30 Min D	4 Santomerse T. 30 Min C	5 Naphthalene (g) 15 Min. D	5 Naphthalene (g) 15 Min. C	5 Naphthalene (g) 30 Min. D	5 Naphthalene (g) 30 Min. C	6 Naphthalene (i) 15 Min D	6 Naphthalene (i) 15 Min C	6 Naphthalene (i) 30 Min. D	6 Naphthalene (i) 30 Min. C
1:200	0	186	0	163	0	17	0	30	2	4	1	4	0	0	0	0	13	0	4	0	39	36	19	21
1:400	0	230	0	201	0	52	0	46	1	1	0	1	0	0	0	0	6	18	8	3	22	49	18	36
1:800	0	156	0	222	0	47	0	47	4	0	3	0	0	0	0	0	0	27	0	5	11	40	7	13
1:1600	0	...	0	...	0	41	0	30	28	8	17	0	0	0	0	0	0	19	0	4	0	34	0	20
No Inactivator or Quaternary	...	182	...	213	...	58	...	52	...	54	..	49	...	62	...	76	...	104	...	57	...	24	...	44

[a] D = Quaternary ammonium detergent (1 cc. of 1,1000 aqueous) + inactivator (10 cc.) + organisms (0.1 cc.).
C = Control. Inactivating agent (10 cc.) + organisms (0.1 cc.).

Klein and Kardon (15), on the other hand, have recently demonstrated that "reversal" of quaternary ammonium action cannot be evidenced once the organisms have been exposed to germicidal concentrations of the compounds. Under certain conditions, the addition of an anionic detergent (sodium decyl sulfate, sodium lauryl sulfate, etc.) to the treated bacterial suspensions merely interrupts the continued action of the cationic detergents, thereby permitting surviving organisms to grow out.

Unlike the ionic reaction between anionic and cationic compounds is the interference of the action of quaternary ammonium germicides by phospholipids. Baker, *et al.* (13), observed the protective action of lecithin, cephalin, and sphingomyelin upon bacteria exposed to the detergents. However, in this instance also, the lipids are ineffective unless they are added to a suspension of organisms before or at the same time as the germicide.

METHOD I

Series I.—In the quantitative chemical method for determining the content of quaternary ammonium compounds in solutions, potassium ferricyanide (16) or potassium thiocyanate may be used to combine chemically with molar equivalents of the germicides. Conceivably, therefore, complete inactivation of the antibacterial action of the compounds may accompany combination of the potassium salts with them. A study was made of the effects of these mixtures in the following manner: Serial dilutions of potassium ferricyanide and potassium thiocyanate were made in distilled water to give final concentrations of 1:200, 1:400, 1:800, and 1:1600. To sterile test tubes containing 10 cc. of the latter solutions was added 1 cc. of a 1:1000 aqueous solution of a quaternary ammonium solution. The contents of the tubes were mixed thoroughly by swirling and allowed to stand at room temperature for five minutes. One-tenth cubic centimeter of a dilute broth culture of *Staphylococcus aureus No. 209* was added to the mixtures as well as to tubes containing the ferricyanide and thiocyanate dilutions but without the germicide. A control tube of an equal volume of distilled water was also included in the test.

Table I, columns 1 and 2, represents the number of colonies in beef extract agar obtained from one loopful of the test mixtures at time intervals of fifteen and thirty minutes.

Results.—From the data given in Table I we note in no instance any evidence of a neutralizing effect of either potassium ferricyanide or potassium thio-

cyanate upon the germicidal effects of the quaternary ammonium germicide. The amounts of the potassium salts used in the more concentrated solutions far exceeded the quantities theoretically required to combine chemically with molar equivalents of the germicides. Potassium ferricyanide and thiocyanate, in the concentrations used, were in themselves devoid of any antibacterial actions.

Series II.—Several commercially available anionic detergents were tested for their ability to inactivate a quaternary ammonium germicide. The compounds included Duponol C, Santomerse T, and several Nacconols (NR, NRSF, SNO). The results of a typical series of tests in which the first two anionic compounds were studied are given in Table I, columns 3 and 4.

Results.—From the data presented in the table, the anionic detergents give a suggestion of a neutralizing effect upon the antibacterial effects of the quaternary ammonium germicide. It is of interest to note that in many instances, while the anionic-cationic mixtures fail to destroy the test organisms completely, i.e., one neutralizing the other, the anionic preparations when used alone in the control prove to be bactericidal for the test organism. Since, however, there was evidence that under the most suitable conditions existing in any one instance, complete inactivation of antibactericidal activity was not accomplished in any of the anionic-cationic mixtures, additional studies were not made on other groups of synthetic anionic detergents.

Series III.—Salts of many of the high molecular weight naphthalene-sulfonic acids and naphthalene-sulfonates are known to cause a marked precipitate when added to solutions of quaternary ammonium salts. Several of the compounds were tested, therefore, for a possible neutralizing action upon the cationic germicides. The naphthalene derivatives studied included the following (*a*) 1-naphthylamine-4-sulfonic acid; (*b*) 1-naphthylamine-5-sulfonic acid; (*c*) 1-naphthylamine-1,5-disulfonic acid; (*d*) 2-naphthylamine-6,8-disulfonic acid; (*e*) 1-naphthylamine-3,6-disulfonic acid; (*f*) 1-naphthylamine-4,6,8-trisodium sulfonate; (*g*) Na-2-amino-8-hydroxynaphthalene-3,6-disulfonate; (*h*) Na-1-hydroxy - 7 - acetylaminonaphthalene - 3,6 - disulfonate; (*i*) Na-symmetric *bis*(*meta*-amino-benzoyl-*meta* - amino - *para* - methyl - benzoyl - 1-naphthylamino-4,6,8-trisulfonic acid) carbamide.

The results of the effects of solutions of two of the most promising compounds (*g* and *i*) upon the germicide are given in Table I, columns 5 and 6.

Results.—Compound (*g*), in the concentrations used, appeared to have a suggestion of a neutralizing action upon the antibacterial effects of the quaternary ammonium germicide. Attention is again pointed to the fact that, as in the results obtained with two anionic detergents, this naphthalene derivative proved to be antagonistic to the test organism when used in the absence of the quaternary compound. Further studies on naphthalene (*g*), therefore, did not appear to be warranted.

Encouraging results were obtained with the naphthalene derivative, (*i*). As evidenced from the data in column 6, the compound more closely approached the requisites for an ideal quaternary ammonium inactivator than did any of the compounds thus far studied. Naphthalene (*i*) in itself showed no evidence of any adverse action against the test organism even when allowed to act for a period of thirty minutes. This compound was studied more extensively as indicated in a later section of the communication.

Series IV.—Soaps are known to have a marked deleterious effect upon the antibacterial effects of quaternary ammonium solutions. This neutralizing action is due to the combining actions of the high molecular weight fatty acid sodium or potassium salts of soap (anionic detergents) with the quaternary ammonium compounds (cationic detergents). In the present study, the effects of castile and U. S. P. green soap were tested against alkyldimethylbenzylammonium chlorides. Certain modifications were made in the test procedure described previously which included the following: One cubic centimeter of the desired concentration of soap solution was placed in a cotton-plugged test tube. To the soap was added 3 cc. of a saline solution (0.85% NaCl) and the mixture autoclaved at fifteen pounds for fifteen minutes. One cubic centimeter of a 1:5000 dilution of the germicide was added to the cool soap-saline solution and immediately inoculated with 0.1 cc. of a 1:500 dilution of *S. aureus*. The contents of the tubes were mixed thoroughly by shaking, and a 4-mm. loopful plated in agar at the end of one hour and again after four hours. The results of this study are presented in Table II.

TABLE II.—EFFECT OF SOAP UPON ANTIBACTERIAL ACTION OF BENZALKONIUM CHLORIDE (1:25,000) AGAINST *S. aureus*—COLONIES PER 4-MM. LOOP

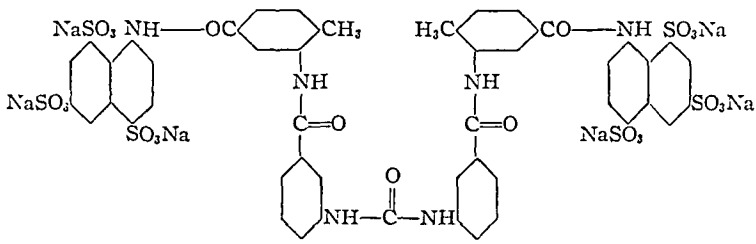
Mg. Soap per 5 Cc.	Inactivator	1:25,000 Quaternary 1 Hr.	1:25,000 Quaternary 4 Hr.	Soap (Only) 1 Hr.	Soap (Only) 4 Hr.
100.00	Castile Soap	20	2	14	0
50.00		22	2	9	0
25.00		41	5	22	0
12.25		39	4	11	1
6.12		82	31	44	9
3.06		83	25	47	6
100.00	U. S. P. Green Soap	3	0	0	0
50.00		3	0	0	0
25.00		5	0	0	0
12.25		18	0	1	0
6.12		23	0	0	0
3.06		5	0	5	0
Control	No Inactivator and No Quaternary	197	97		
		168	116		

Results.—From the data given in the table, it is apparent that neither castile nor U. S. P. green soap can be used as neutralizer for the quaternary ammonium germicide. While both soaps show evidence of an action against the germicide, this effect

is not sustained for any length of time and, furthermore, both agents in themselves are bactericidal for the test organism.

METHOD II

With the evidence presented thus far the most promising data obtained in the series of compounds studied were the action of naphthalene (*i*) (Series III) upon the quaternary ammonium germicide. The compound is the sodium salt of symmetric *bis*-(*meta*-amino-benzoyl-*meta*-amino-*para*-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonic acid) carbamide and has the following structural configuration:



It occurs as a white powder which dissolves readily in cold water. Aqueous solutions are neutral in reaction and remain colorless even after autoclaving. Upon standing at room temperature for a period of several weeks, however, the solutions may assume a pink to deep red color.

The compound was first described by Heymann, Kothe, *et al.* (17) as a specific chemotherapeutic agent for the prophylaxis and treatment of African sleeping sickness caused by trypanosomes. The drug is known in the United States as "Suramin Sodium" (U. S. P. XIII) and in certain foreign countries as "Germanin" or "Bayer 205."

Tiedeman, Fuchs, *et al.* (18), have proposed a method for controlling sanitation of food utensils. In the procedure, use is made of swab containers containing 4 cc. of sterile 0.1% normal sodium thiosulfate solution (chlorine inactivator) buffered at *p*H 7.2. One cubic centimeter of the solution is used for each utensil to be swabbed, i.e., 4 cc. for four utensils. In the present study, Suramin Sodium was evaluated as an inactivator for quaternary ammonium germicides in the presence of the buffer solution in the following manner. To a series of tubes were added 3 cc. of buffer and 1 cc. of a dilution of Suramin Sodium. The tubes were autoclaved at fifteen pounds for fifteen minutes and upon cooling to each was added 1 cc. of a 1:1000 aqueous solution of Benzalkonium Chloride, *p*-tertiaryoctylphenoxyethoxyethyldimethylbenzylammonium chloride or cetylpyridinium chloride, and 0.1 cc. of a 1:500 saline dilution of *S. aureus*. The contents of the tubes were mixed thoroughly by swirling and allowed to stand at room temperature. Table III presents the number of colonies in beef extract agar obtained from one loopful of the test mixtures when sampled at the end of one hour and again after four hours.

Results.—It is evident from the data given in Table II that within the limits of error in the sampling of the test solutions Suramin Sodium will definitely inactivate the antibacterial actions of the three quaternary ammonium compounds. Furthermore, no appreciable differences in counts were noted between the one-hour and four-hour test periods on the samples containing the higher concentrations of the inactivator.

METHOD III

In the methods described thus far, the inactivator and quaternary ammonium compounds were mixed just prior to the addition of the test organisms. It appeared desirable, therefore, to determine the neutralizing effects of Suramin Sodium upon a germicide after the latter had been allowed to act

TABLE III.—INACTIVATION OF BENZALKONIUM CHLORIDE (I), *p*-TERTIARYOCTYLPHENOXY-ETHOXYETHYLDIMETHYLBENZYLAMMONIUM CHLORIDE (II), AND CETYL PYRIDINIUM CHLORIDE (III) AGAINST *S. aureus*—COLONIES PER 4-MM. LOOP

Mg. Suramin Sodium per 5 Cc.	1:5000 Quaternary Ammonium Compound					
	I		II		III	
	1 Hr.	4 Hr.	1 Hr.	4 Hr.	1 Hr.	4 Hr.
50	280	258	263	219	151	158
40	213	319	290	165	77	54
30	248	252	209	180	85	54
20	230	263	237	93	50	48
10	190	203	231	55	47	7
5	166	94	162	71	16	3
2.5	100	32	252	28	24	0
1	175	166	171	58	126	79
Control—No Inactivator No Quaternary	308	320	293	274	372	344

TABLE IV—ACTION OF SURAMIN SODIUM UPON BENZALKONIUM CHLORIDE AFTER LATTER HAS BEEN ACTING UPON *S. aureus*—COLONIES PER 4-MM. LOOP

Quaternary Dilution per 1 Cc.	Inactivator—0.05 Gm. per 4 Cc. Culture Dilution 1:500[a]	Inactivator—0.05 Gm. per 4 Cc. Culture Dilution Undiluted	No Inactivator Culture Dilution 1:500	No Inactivator Culture Dilution Undiluted
1:1,000	0	0	0	0
1:5,000	0	0	0	0
1:10,000	0	190	0	9
1:20,000	0	630	0	45
1:30,000	0	704	0	524
1:40,000	0	+++	0	++
1:50,000	3	++++	0	+++
1:60,000	5	++++	0	++++
1:70,000	4	++++	0	++++
1:80,000	46	++++	25	++++
1:90,000	66	++++	5	++++
1:100,000	68	++++	24	++++
None	300	++++	130	++++

[a] 1:500 = 0.1 cc. of 1:500 culture of *S. aureus* to 1 cc. Benzalkonium chloride.
Undiluted = 0.1 cc. of undiluted culture of *S. aureus* to 1 cc. Benzalkonium chloride.
++ to ++++ = Too many to count, yet numbers may be less (++, +++) than those noted in many of the other plates showing maximum number of colonies (++++).

upon the bacteria. This was carried out as follows: To 1-cc. quantities of a series of dilutions of Benzalkonium Chloride was added 0.1 cc. of a 1:500 saline dilution of *S. aureus*. A second series received 0.1 cc. of an undiluted broth culture of the organisms. The mixtures were allowed to stand for thirty minutes at which time 4 cc. of a 1:80[1] dilution of Suramin Sodium was added to each tube. The contents of the tubes were mixed thoroughly and again allowed to stand for thirty minutes. A 4-mm. loopful of the test suspensions was then plated in agar and incubated as before. The results of this study are presented in Table IV.

Results.—In summarizing Table IV the following may be noted: In the absence of Suramin Sodium the limiting dilution of the quaternary ammonium germicide that apparently destroyed the dilute suspension (1:500) of test organisms was 1:70,000. In the presence of the inactivator, however, complete absence of growth was first noted in the test medium containing 1:40,000 of the cationic detergents. Yet dilutions in the latter series up to and including 1:70,000 showed evidence of only a few organisms in the test sample. This again emphasizes the high bactericidal activity of quaternary ammonium germicides.

In the series inoculated with 0.1 cc. of undiluted culture of *S. aureus*, complete absence of growth was noted in the presence or absence of the inactivator in the 1:5000 dilution of Benzalkonium Chloride. The neutralizing action of the inactivator upon the germicide is again evidenced by the greater number of colonies that developed in its presence as compared to the relatively few that grew out in the 1:10,000 and 1:20,000 samples of the germicides in the absence of neutralizer.

[1] 1:80 = 0.0125 Gm./cc., or 0.050 Gm./4 cc. of solution.

In studies entirely independent from these, Gilcreas (19) found that Suramin Sodium would effectively neutralize a quaternary ammonium germicide in the presence of *Escherichia coli* and *Staphylococcus albus* as test organisms. Furthermore, this inactivation was sustained for a period of twenty-four hours. Favorable results on the use of the inactivator have also been obtained by Jamieson (20), Weber and Black (21), and Mueller, Seeley, and Larkin (22).

SUMMARY

A résumé has been presented on the necessity for providing a suitable means for inactivating the high antibacterial activity of quaternary ammonium germicides.

Synthetic anionic detergents, as well as soaps, fail to completely inactivate the surface-active cationic detergents.[2]

A group of sulfonates and sulfonic acids were studied for their possible application as inactivators for the germicides. Suramin Sodium, a sulfonic acid derivative, more closely approached the requirements for an inactivator for quaternary ammonium compounds.

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[2] Quisno, Gibby, and Foter (23) in a recent publication appearing after the present study had been completed, described a medium containing lecithin and "Tween 80" (a polyoxyethylene derivative of sorbitan monooleate) for the inactivation of relatively high concentrations of quaternary ammonium salts. When used alone the medium was also found to promote the growth of some strains of bacteria.

New Syntheses of Caffeine and Theophylline*

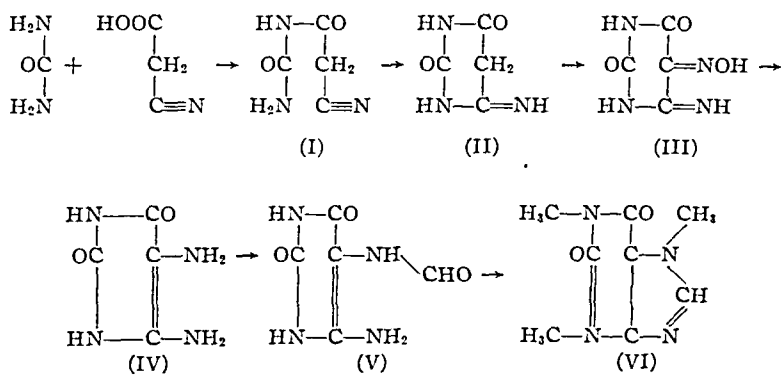
By BOGUSLAW BOBRANSKI and ZDZISLAW SYNOWIEDSKI†

Starting with urea and cyanacetic acid, caffeine and theophylline have been prepared by convenient modifications of methods previously described for these syntheses.

After its structure was established, a synthesis of caffeine was described by E. Fischer (1). This synthesis, from dimethylurea and malonic acid dichloride, was important only for its theoretical value. For practical use, it is preferable to start with uric acid which is now produced from guano.[1]

As a result of war conditions in 1942 there was an extreme shortage of caffeine in Poland. In order to provide caffeine it was necessary to develop a convenient and economical synthesis of this alkaloid. In connection with this problem the Traube (2) synthesis of caffeine from mono- or dimethylurea was considered. To avoid the inconvenience of producing these substances, urea was used as the starting product and the methyl groups were introduced in the further stages of the synthesis. Cyanacetic urea (I) was produced by condensation of urea with cyanacetic acid in the presence of acetic anhydride (3) which was immediately transformed by action of 40 per cent sodium hydroxide into 4-imino-2,6-dioxyhexahydropyrimidine (II). The latter, without being isolated, was converted by action of nitrous acid into iminovioluric acid (III).

The iminovioluric acid (III) was reduced by means of zinc powder and sulfuric acid (4) to the sulfate of the diamine (IV). This base was converted by heating with anhydrous sodium formate and formic acid into the 4-amino-5-formylamino-2,6-dioxypyrimidine (V), which was isolated. The direct methylation of (V) with 2 moles of dimethylsulfate did not yield the expected 1,3-dimethyl derivative, but a mixture of dimethyl derivatives. Upon heating the solution containing this mixture with sodium hydroxide and then adding an additional mole of dimethyl sulfate, caffeine (VI) was formed.



Thus (V) in a simple procedure was converted directly into caffeine (VI). The complete synthesis of caffeine consists consequently of four operations: (*a*) the preparation of iminovioluric acid (III); (*b*) the preparation of 4,5-diamino-2,6-dioxypyrimidine sulfate (IV); (*c*) the formylation of the diamine; (*d*) the preparation of the caffeine from the formylamine.

Efforts to synthesize theophylline by a method analogous to that used for caffeine were unsuccessful. Neither could 4-imino-5-formylamino-2,6-dioxypyrimidine (V) be converted into the 1,3-dimethyl derivative

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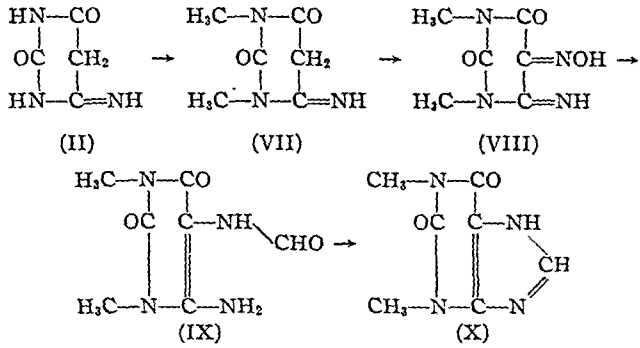
[1] For a review of methods of synthesis of caffeine see H. Schwaneberg, *Pharm. Ind.*, 10, 109–11(1943).

with a satisfactory yield. The sodium salt of 4-imino-2,6-dioxy-hexahydropyrimidine (II), however, was successfully methylated with dimethyl sulfate at 0°, a reaction not previously accomplished, as is mentioned by Max Conrad (5). The 1,3-dimethyl-4-imino-2,6-dioxyhexahydropyrimidine (VII) obtained by this reaction was converted into 1,3-dimethyliminovioluric acid (VIII) and the latter by treatment with zinc and formic acid transformed directly into the 1,3-dimethyl - 4 - amino - 5 - formylamino - 2,6-dioxytetrahydropyrimidine (IX) which is converted into theophylline (X) by heating in a sodium hydroxide solution (6). Thus the synthesis of theophylline was realized in 5 operations:



EXPERIMENTAL

Preparation of Iminovioluric Acid.—A mixture of 950 Gm. of chloracetic acid and 300 cc. of water was neutralized with a 40% solution of sodium hydroxide (about 750 cc.) using phenolphthalein as the indicator. A solution of 510 Gm. of sodium cyanide in 1 L. water at a temperature of 50° was added to this mixture, and the temperature was kept below 80° during the reaction. The mixture, at the end of the reaction, was cooled to 30° and acidified with 30% sulfuric acid (about 1.55 Kg.) using Congo red as indicator. To the cold cyanacetic acid solution was added 600 Gm. of urea and the resulting solution (about 5 L.) evaporated under diminished pressure to dryness. The residue was cooled, 1500 cc. of acetic anhydride was added, and the mixture was stirred from time to time and cooled so that the temperature did not rise above 70°. The reaction product was allowed to stand overnight, after which the acetic acid formed was removed completely by distillation under diminished pressure. To the dry residue 2.3 L. of a 40% solution of sodium hydroxide was added with stirring, the temperature being maintained below 60° during the reaction. After two to three hours the sodium salt of 4-imino-2-6-dioxyhexahydropyrimidine separated from the reaction mixture. Then 725 Gm. of sodium nitrite dissolved in 3 L. water was added, and into this mixture a 10% sulfuric acid solution was introduced to obtain a faintly acid reaction with litmus and neutral reaction with Congo red. A red microcrystalline iminovioluric acid was obtained which, after standing for twelve hours, was removed by filtration and washed with 1500 cc. of water. Yield: 1 Kg.; 64%.

Preparation of 4,5-diamino-2,3-dioxypyrimidine sulfate.—One kilogram of iminovioluric acid was suspended in 20 L. of 20% sulfuric acid. The mixture was stirred vigorously and 1 Kg. of zinc powder was introduced in small portions with cooling so that the temperature did not rise higher than 30°. The stirring was continued during one hour, the mixture cooled, and the crude 4,5-diamino-2,3-dioxypyrimidine sulfate filtered off, washed with 0.5 L. water, and dried at 40°. Yield: 1 Kg.; 76%.

Preparation of 4-Amino-5-formylamino-2,6-dioxypyrimidine.—One kilogram of dry, crude 4,5-diamino-2,6-dioxypyrimidine sulfate was mixed thoroughly with 0.6 Kg. of dry sodium formate, 5 L. of formic acid was added, and the mixture was heated during four to five hours on a steam bath under a reflux condenser. After standing for twelve hours at room temperature the crude formyl derivative was removed by filtration, washed several times with water and finally dried. Yield: 750 Gm.; 90.5%.

Preparation of Caffeine.—Seven hundred fifty grams of finely powdered, crude 4-amino-5-formylamino-2,6-dioxypyrimidine was introduced into a round-bottomed flask containing 2.95 L. of 3 *N* sodium hydroxide solution, 1.12 Kg. of dimethyl sulfate was added with stirring, the temperature being maintained below 40°. After all the dimethyl sulfate was added the stirring was continued for one and one-half hours and then 1.39 L. of 3 *N* sodium hydroxide solution was added. The reaction mixture was heated rapidly to 90° and the stirring continued another half hour. After cooling to room temperature, 0.57 Kg. of dimethyl sulfate was added slowly with stirring. After one hour the mixture was filtered and the filtrate was concentrated

under diminished pressure to 3 L. Upon standing for forty-eight hours the crude caffeine (about 300 Gm.) crystallized and was removed by filtration. From the filtrate, by extracting 5 times with 200 cc. of chloroform, an additional 150 Gm. of crude caffeine was obtained. The crude caffeine was recrystallized 4 times from water, the hot solutions being decolorized with charcoal. Yield: 350 Gm.; 37.5%. The over-all yield of caffeine was 16.5% of theory based on the starting product.

Preparation of 4-Imino-2,6-dioxyhexahydropyrimidine.—This product was obtained by the procedure outlined for the preparation of iminovioluric acid. The sodium salt of 4-imino-2,6-dioxyhexahydropyrimidine which is formed intermediately by condensation of cyanacetylurea by means of sodium hydroxide at 60° was dissolved in 8 L. of water and the crude 4-imino-2,6-dioxyhexahydropyrimidine was precipitated by acidification of the solution with acetic acid. Yield: 710 Gm.; 56%.

Preparation of 1,3-Dimethyl-4-imino-2,6-dioxyhexahydropyrimidine.—Seven hundred ten grams of 4-imino-2,6-dioxyhexahydropyrimidine was suspended in 3.73 L. of 3 *N* sodium hydroxide solution. After cooling to 0°, 1.58 Kg. (1.19 L.) of dimethyl sulfate was added and the mixture stirred vigorously for five hours at 0°. The crystalline 1,3-dimethyl derivative formed was filtered off. From the filtrate upon acidification with acetic acid the unchanged 4-imino-2,6-dioxyhexahydropyrimidine was recovered and methylated as described above. Yield: 530 Gm.; 61%.

Preparation of 1,3-Dimethyliminovioluric acid.—Five hundred thirty grams of 1,3-dimethyl-4-imino-2,6-dioxyhexahydropyrimidine was dissolved in 16 L. of boiling water, 260 Gm. of sodium nitrite was added, and the hot solution was treated with 270 Gm. of 90% acetic acid. Upon cooling, the purple-colored crystalline 1,3-dimethyl-4-iminovioluric acid was collected on a filter and washed with about 1 L. of water. Yield: 530 Gm.; 84.5%.

Preparation of 1,3-Dimethyl-4-amino-5-formylamino-2,6-dioxytetrahydropyrimidine.—Five hundred thirty grams of 1,3-dimethyl-4-iminovioluric acid was dissolved in 5.3 Kg. of 95% formic acid and to this solution, while being stirred rapidly, 430 Gm. of zinc powder in small portions was added. The reaction mixture was cooled to 30°. As soon as the red color of the solution had disappeared (after two to three hours) the reaction mixture was refluxed for three hours. Upon cooling the zinc formate separated, was filtered off, and washed with 0.5 Kg. of 95% formic acid. From the filtrate the formic acid was removed by distillation under diminished pressure. The residue was dissolved in 8 L. of water at 80–90° and filtered from the insoluble zinc formate. A yield of 265 Gm. of 1,3-dimethyl-4-amino-5-formylamino-2,6-dioxyhexahydropyrimidine crystallized from the filtrate. The mother liquor upon concentration to 1 L. yielded an additional 120 Gm. of the same product. Yield: 385 Gm.; 62.5%.

Preparation of Theophylline.—Three hundred eighty-five grams of 1,3-dimethyl-4-amino-5-formylamino-2,6-dioxytetrahydropyrimidine was suspended in 3.85 L. of hot water and 310 cc. of a 40% solution sodium hydroxide was added. The solution was heated on a water bath to 90° for ten minutes and upon cooling 230 cc. of concentrated hydrochloric acid was added. The still alkaline solution was treated with 30% acetic acid to weakly acid reaction (about 14 cc.). The theophylline crystallized after some hours, was filtered off, and washed with 300 cc. of water. The crude product (300 Gm.) was dissolved in 6 L. of boiling water and the solution decolorized with charcoal and allowed to stand for several hours for crystallization. Two hundred eighty grams of pure theophylline was obtained (80% theoretical). The over-all yield of theophylline was 14.3%.

SUMMARY

1. A new method for the synthetic preparation of caffeine from urea and chloracetic acid is described.

2. The conversion of 4-amino-5-formylamino-2,6-dioxypyrimidine into caffeine is described.

3. The methylation of 4-amino-2,6-dioxyhexahydropyrimidine in position 1 and 3 by means of dimethyl sulfate is described. Thus a simple method for the preparation of theophylline from urea and chloracetic acid as starting materials has been developed.

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The Fractionation by Alcohol of Equine Antitoxic Plasma*

By ALFRED H. FOWELL and FREDERICK F. JOHNSON

The desirability of concentrating equine antitoxin relatively free from pyrogen prompted an investigation of the use of alcohol fractionation at reduced temperatures. In normal antitoxic plasma the antitoxin is found in the T-globulin and gamma-globulin components. In pepsin digested antitoxic plasma the antitoxin is found in a modified component formed by the merger of the "T" and "gamma." By the alcohol fractionation methods described, either the "T + gamma" or the new modified component can be obtained in a satisfactory state of purity as shown by electrophoretic analyses. The methods have been applied to the preparation of diphtheria and vibrion septique antitoxin.

VARIOUS workers (1–4) have reported that normal horse plasma can be separated electrophoretically into the following six well-characterized plasma proteins: albumin, α_1-globulin, α_2-globulin, β-globulin, fibrinogen, γ-globulin. The concept that most antibodies are found associated with the γ-globulin fraction has been repeatedly verified in recent years. Van der Scheer and co-workers (5, 6) reported that hyperimmunized horse plasma contains an additional electrophoretic component which they termed T-globulin and which exhibited an electrophoretic mobility between that of the β-globulin and γ-globulin. They concluded that although the T-globulin "peak" became much larger when a large amount of antitoxin appeared in the serum, the area of the peak could not be taken as proportional to the measured antitoxin activity. Nevertheless, the T-globulin undoubtedly contains the major portion of the antitoxin of a hyperimmune serum or plasma.

The usual processes for preparing antitoxin for clinical administration employ a partial enzyme digestion of the horse protein. This digestion has been conducted at temperatures ranging from 0° to 60° (7–9). Under most conditions, the antitoxin is more stable to enzyme action than is the inert protein; thus an enzyme digestion step, when used in conjunction with the usual procedures for purifying antitoxin by salt fractionation, generally results in a greatly increased purification. Coghill, *et al.* (10), reported that enzyme (Takadiastase) digestion so alters the antitoxin that its horse protein specificity is greatly reduced.

Van der Scheer and Wyckoff (11, 12) followed the course of peptic digestion of tetanus antitoxic horse serum with electrophoretic analyses and showed that during digestion the T-globulin is rapidly converted into a slower moving component of mobility similar to that of γ-globulin; in other words, the T- and γ-globulins are said to merge. Deutsch, *et al.* (13), working with human γ-globulin, have reported conditions for peptic digestion which result in splitting the γ-globulin molecules into half molecules without destroying the antibody activity.

The fractionation of human plasma proteins by employing isoelectric precipitation with varying conditions of ethanol concentration and ionic strength at reduced temperatures has been described by Cohn (14). The conditions for separation and purification of human γ-globulin have been further developed by Deutsch (15) who employed ionic strengths as low as 0.01. Smith and Gerlough (16) applied the Cohn (14) fractionation scheme to hyperimmune tetanus horse plasma and found the antitoxic activity to be distributed between at least three distinct proteins: T-globulin, γ-globulin, and a β-globulin.

We have attempted to devise a set of conditions where, by varying the *p*H, ionic strength, temperature, and ethanol content of horse plasma, the antitoxic globulins can be removed in a state of suitable purity. Cold alcohol fractionation methods are

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easily applicable to large-scale preparation, and they can be applied with less risk of pyrogen formation than the conventional salt fractionation procedures. We have applied alcohol fractionation to both pepsin digested and undigested horse plasma and have studied the pepsin digestion of antitoxic fractions subsequent to their separation. Whether or not the enzyme digestion of an equine antitoxin actually results in a lower incidence of "serum sickness" is in our opinion still open to question, since the reported clinical comparisons generally have been performed with undigested antitoxin of average potency and digested antitoxin of higher potency. Since the alcohol fractionation methods can yield antitoxin of potency equivalent to that obtained from salt enzyme procedures, it should be possible to compare clinically the effects of digested and undigested antitoxin of equal horse protein content per dose.

EXPERIMENTAL

Methods.—The antitoxic plasmas were prepared by usual methods, were of low to average potencies, and were taken from pools each representing five or more horses bled in a citrate anticoagulant. Diphtheria antitoxin assays were made by the Ramon flocculation technique (17) except where otherwise stated.

The fractionation conditions of *p*H and ionic strength were measured or calculated, respectively, on the basis of the volume obtained after the addition of alcohol. The *p*H of alcoholic solutions was determined on undiluted aliquots at 10°. Alcohol concentrations are expressed as per cent by volume. When adding alcohol to an aqueous protein solution or one of low alcohol content, the heat of dilution was minimized by previously diluting the alcohol to 50% and cooling to below —15°. All alcohol additions were gradual with vigorous stirring.

Electrophoretic analyses were made with the standard Tiselius apparatus using the Longsworth scanning device. The runs were made at 0.7° using a diethylbarbiturate buffer of *p*H 8.6 and ionic strength 0.10.

All protein precipitates were analyzed after being dried from the frozen state and being dissolved in 0.9% sodium chloride between *p*H 7.0–7.4.

Fractionation of Heat-Defibrinated Plasma.—Antidiphtheria horse plasma was held at 56° for fifteen minutes. The precipitate was removed by filtration and the filtrate was divided into four 45-cc. aliquots each of which was diluted with water to a volume of 100 cc. Four successive precipitates were formed from each aliquot under the conditions given in Table I. For example, from aliquot I, precipitate IA was formed and removed, then precipitate IB was formed from the effluent and removed, etc. The plasma used had a potency of 650 Lf/cc. The best precipitate in this group was IIIB which had a potency of 14,240 Lf/Gm. and contained 67% cent of the original unitage.

TABLE I.—FRACTIONATION OF HEAT DEFIBRINATED PLASMA

Plasma	*p*H	EtOH, %	Temp., °C	Dry Wt., Gm.	Units	Units/Gm.
IA	6.28	16	0	0.7956	2,387	3,000
IB	6.28	19	−2	1.124	15,400	13,700
IC	6.25	22	−3	0.2698	3,632	13,460
ID	6.28	25	−5	0.1265	1,054	8,333
IIA	6.40	16	0	0.6849	1,750	2,500
IIB	6.53	19	−2	1.199	15,490	12,910
IIC	6.54	22	−3	0.4193	7,425	17,700
IID	6.52	25	−5	0.0961	837	8,721
IIIA	6.79	16	0	0.6727	2,018	3,000
IIIB	6.85	19	−2	1.378	19,610	14,240
IIIC	6.81	22	−3	0.1330	2,163	16,260
IIID	6.82	25	−5	0.0715	392	9,677
IVA	7.48	16	0	0.2530	781	3,086
IVB	7.48	19	−2	1.333	15,620	11,790
IVC	7.55	22	−3	0.2586	2,068	8,000
IVD	7.52	25	−5	0.1294	1,078	8,333

Fractionation of Unmodified Plasma.—Antidiphtheria horse plasma, without defibrination or enzyme digestion, was fractionated by manipulation of the four variables (ethanol concentration, *p*H, ionic strength, temperature) listed in Table II. Fractions 1 through 3 were obtained as successive precipitates from one aliquot; fractions 4 through 7 were obtained as successive precipitates from another aliquot. Fractions 8 through 11 were obtained from four aliquots of the effluent from fraction 7, and in each case consisted of essentially all T-globulin and γ-globulin. The units in Table II were obtained by the standard guinea-pig protection test.

A study of the results shown in Table II indicated that the best approach, within the range of conditions studied, would be to discard a precipitate

formed under the conditions of fraction 6, and then to precipitate the antitoxin under the conditions of fraction 11. Further experimentation involving minor variations of these conditions led to the construction of what we term Method 1, the method being applicable to the fractionation of whole undigested antitoxic horse plasma. Electrophoretic patterns of the products obtained by Method 1 show them to consist of T-globulin plus smaller amounts of γ-globulin and β-globulin. A typical electrophoretic pattern is shown in Fig. 1. By Method 1, an antidiphtheria plasma of low potency (ca. 400–500 u./cc.) yields about 65% of the antitoxin of a potency of about 15,000 u./Gm. An antidiphtheria plasma of average potency (ca. 600–700 u./cc.) yields about 80% of the antitoxin of a potency of about 20,000 u./Gm.

Statement of Method 1.—Dilute each liter of plasma with about 1900 cc. of 1° water. By the use of cold 50% ethanol, *p*H 4 acetate buffer, and sodium chloride, adjust to the following conditions: ethanol 12%, *p*H 5.10, ionic strength 0.057, temperature —2°. Remove the precipitate by centrifugation at —2° and discard. Filter the effluent and adjust to *p*H 7.4 with 2 *N* NaOH. By the use of cold 95% ethanol, adjust to the following conditions: ethanol 34%, *p*H 7.40, ionic strength 0.059, temperature —5°. Remove the precipitate by centrifugation at —5°, suspend in a minimum quantity of water, and dry by vacuum from the frozen state.

Experiments on Enzyme Digestion.—A 2% solution of antitoxin (19,200 u./Gm.) prepared by Method 1 was subjected to pepsin digestion at low temperatures and low *p*H. Variations of time, amount of pepsin, temperature, and *p*H between 2.0 and 4.0 were tried, but in all cases a loss of activity approximating 50% resulted.

A comparison was made of the pepsin digestion of the same 2% solution of antitoxin and the pepsin digestion of whole plasma diluted to 2% protein, the original citrated horse plasma being 7% protein. The conditions were: temperature 2–5°, *p*H 2.4, 1:10,000 pepsin 0.2%. Samples were removed at intervals for unitage assay by the Ramon flocculation test. The potency of the 2% antitoxin prepared by Method 1 decreased approximately 50% in thirty

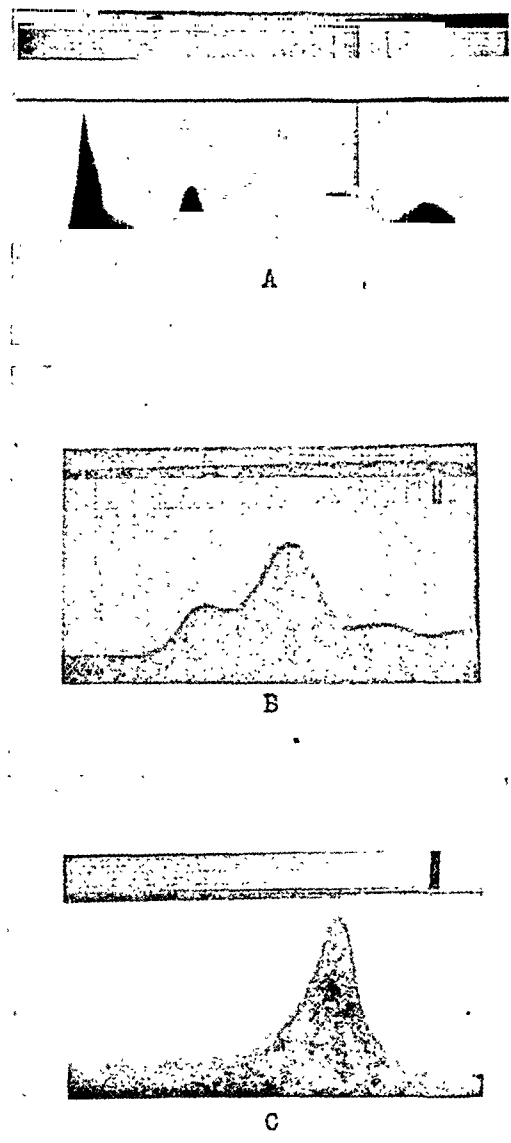

Fig. 1.—Electrophoretic patterns of (A) diphtheria horse plasma, (B) diphtheria antitoxin made by method 1, and (C) diphtheria antitoxin made by method 3.

TABLE II.—FRACTIONATION OF UNMODIFIED PLASMA

	Fraction						Electrophoretic Analysis, %					
	Ethanol Conc., %	*p*H	Γ/2	Temp., °C.	% Yield of Units	Units/Gm.	Alb.	α-Glob.	β-Glob.	Fib.	T	γ-Glob.
1	14	5.1	0.04	−3	55	10,200	6.1	8.1	7.0	[a]	42.8	36.0
2	25	7.2	0.03	−5	7.6	23,300	4.4	12.2	0	0	73.6	9.8
3	40	4.8	0.02	−5	23	4,300	56.8	27.8	0	0	15.3	0
4	S	5.1	0.05	−2	0	0	0	3.9	19.9	76.1	0	0
5	10	5.1	0.05	−3	5.4	4,320	8.7	6.3	0	0	33.6	51.3
6	12	5.1	0.05	−3	11	7,060	...	...	...	...	...	...
7	14	5.1	0.05	−3	26	18,600	8.3	15.0	0	0	54.6	22.1
8	34.8	6.8	0.05	−5	67	17,300						
9	34.6	7.0	0.05	−5	74	17,800						
10	34.6	7.2	0.05	−5	65	17,700						
11	33.8	7.4	0.05	−5	55	21,500						

[a] The fibrinogen in fraction 1 was masked by the T-globulin; the concentration of fibrinogen is therefore included in the concentration ratio for T.

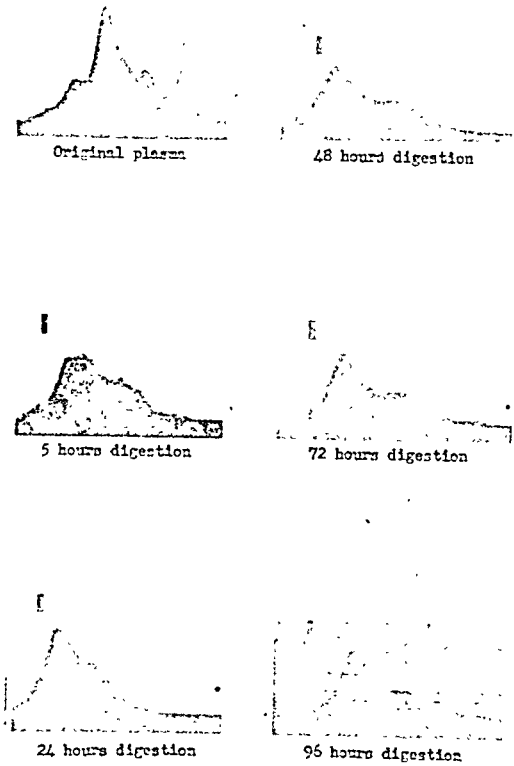

Fig. 2.—Electrophoretic patterns of pepsin-digested diphtheria horse plasma.

minutes and then continued to decrease slightly, whereas the potency of the plasma diluted to 2% protein decreased only 30% in thirty minutes and showed no further decrease during 150 minutes.

The pepsin digestion of diluted antidiphtheria plasma was then studied at three higher *p*H values. In each case, plasma was diluted with 2.6 volumes of water, adjusted to *p*H 6.0 with hydrochloric acid, 0.36 Gm. 1:10,000 pepsin added per 100 cc. of plasma adjusted to the indicated *p*H with hydrochloric acid, and maintained at 2° during digestion. The results in terms of potency and flocculation time are shown in Table III. The results of these digestion studies clearly indicate that within the *p*H range of 3.5 to 4.0 and at a temperature of 2°, the flocculating activity remains constant after one hour, but that a considerable period is required for the flocculating time to drop to a proper value. A low Kf is taken as an indication of sufficient digestion, resulting in the merger of the T and gamma components and presumably the splitting of the antitoxin into half molecules. The merger of these two antitoxin bearing components as measured against time of digestion is exemplified in Fig. 2 which shows the electrophoretic patterns of plasma (diluted with one volume of water) and digested at *p*H 4.0, temperature 2°, for various time intervals. This digestion series was also followed with measurements of the Lf units per cc. and the corresponding Kf values and the results roughly verified those reported in Table III for *p*H 4.00.

Some of the products of the enzyme digestion studies just described were submitted to an arbitrary cold alcohol fractionation, namely, discarding the *p*H 5.1, 15% ethanol, —2° precipitate, and saving the subsequent *p*H 7.4, 35% ethanol, —5° precipitate. Although none of the final fractions were of adequate potency and yield, it was noted that the potency markedly increased with increasing dilution of the plasma before digestion. A plasma originally diluted to about 1 to 7 appeared to allow the most satisfactory fractionation after digestion. On the other hand, a plasma diluted 1 to 7 would never show a merger of the T and gamma components or a low Kf value after digestion for as long as five days at 2° and *p*H 4.0. Apparently the lowering of the ionic strength by diluting the plasma 1 to 7 greatly increased the sharpness of fractionation but prevented an adequate degree of digestion. For this reason, subsequent pepsin digestions were made on plasma diluted 1 to 2, with the dilution being increased after digestion to 7 times the plasma volume.

Fractionation of Pepsin Digested Plasma.—Using diluted antidiphtheria plasma, digested as described above, a large number of fractionations were performed to find suitable conditions for the precipitation of an inert fraction 1. (Conditions for precipitation of the fraction 2 antitoxin could be obtained rather closely from previous data.) The pertinent data for the discard fraction is shown in Table IV. In this case, antidiphtheria plasma was diluted 1 to 2 with water, adjusted to *p*H 6.0, treated with 0.36 Gm. 1:10,000 pepsin per 100 cc. plasma, adjusted to *p*H 4.0 and maintained at 2° for ninety-six hours. The mixture was clarified by continuous Sharples centrifugation (15,000 r.p.m.) at 2° and the effluent was diluted to aproximately seven times the plasma

TABLE III.—PEPSIN DIGESTION OF DILUTED PLASMA

*p*H	Digestion, Hr.	Lf Units/Cc.	Kf, Min.
3.50	0	136	22
	1	104	10
	2	104	11
	3	104	8
	4	104	8
	5	104	6
3.75	0	124	26
	0.5	104	18
	16	104	14
4:00	0	149	20
	0.5	124	18
	1	124	18
	2	124	17
	3	124	17
	4	124	17
	24	124	14
	48	124	10
	72	124	7
	96	124	7

volume. A series of aliquots were adjusted to the conditions of *p*H and ethanol concentration shown in Table IV, the temperature being kept constant at —2° and the ionic strength at 0.015.

From Table IV, the *p*H 4.6, ethanol 20%, —2°, ionic strength 0.015 fraction was selected as a discard fraction affording a satisfactory compromise between loss of inert material and loss of unitage. Subsequent precipitation from the effluent of the fraction 1 at *p*H 7.0, 30% ethanol, and —5° yielded satisfactory antitoxin. The method was tentatively designated as Method 2 and was used in a series of production runs on pyrogenic antidiphtheria and anti-vibrion-septique plasma. In all such runs, antitoxin of a low pyrogenicity was obtained in yields averaging 53% of the starting unitage.

The chief disadvantage of Method 2 is the large volumes which must be handled since the low ionic strength is attained by dilution. This disadvantage can be overcome by precipitating a total globulin fraction and redissolving it in salt-free water to an appropriate volume.

The conditions for precipitating a total globulin fraction were taken from the data in Table V. Antidiphtheria plasma was pepsin digested in the usual manner and aliquots were brought to the fractionation conditions of Table V with a constant temperature of —5° and a constant ionic strength of 0.07. The per cent yields of activity are based on the activity of the original plasma which loses approximately 30% of its activity during pepsin digestion.

The conditions selected for the total globulin precipitation were: *p*H 6.6, ethanol 35%, temperature —5°, ionic strength 0.07. A large total globulin fraction was prepared by these conditions and was redissolved in sufficient 2° water to bring the ionic strength to approximately 0.027. By adjusting the *p*H to 5.1 at +2°, a large amount of low potency material (2500 Lf u./Gm.) separated and was discarded by centrifugation. This first discard fraction was designated fraction 1*a*. Equal aliquots of the effluent from fraction 1*a* were then adjusted to the fractionation conditions described in Table VI at a constant ionic strength of 0.017.

TABLE IV.—PRECIPITATION OF DISCARD FRACTION FROM PEPSIN DIGESTED ANTIDIPHTHERIA PLASMA AT —2° AND IONIC STRENGTH 0.015

Fraction			
*p*H	EtOH, %	Yield, Gm.	Lf Units/Gm
4.2	20	0.3301	<1182
4.2	25	0.3708	<1610
4.2	35	0.3708	<1610
4.6	10	0.1499	<1240
4.6	15	0.2796	<1331
4.6	20	0.4279	1766
4.8	10	0.1940	< 960
4.8	15	0.3684	1234
4.8	20	0.5438	2933
5.0	10	0.2410	<1079
5.0	15	0.4225	2359
5.0	20	0.5809	4625

TABLE V.—PRECIPITATION OF TOTAL GLOBULIN FRACTION FROM PEPSIN DIGESTED ANTIDIPHTHERIA PLASMA AT —5° AND IONIC STRENGTH 0.07

Fraction		% Yield	
*p*H	EtOH, %	of Units	Lf Units/Gm.
6.6	30	65.6	7194
7.0	30	62.1	7291
7.2	30	62.8	7316
6.6	35	68.3	7071
6.8	35	62.4	7088
7.0	35	62.9	7203
7.2	35	59.5	6930

TABLE VI.—PRECIPITATION OF SECOND DISCARD FRACTION (1*b*) FROM PEPSIN DIGESTED ANTI DIPHTHERIA PLASMA AT IONIC STRENGTH 0.017

Fraction				
*p*H	EtOH, %	Temp., °C.	Yield, Gm.	Lf Units/Gm
4.4	5	—1	0.4200	3500
4.6	5	—1	1.006	5404
4.8	5	—1	1.474	6194
4.4	10	—1	0.9501	4010
4.6	10	—1	1.425	6090
4.8	10	—1	2.053	6679
4.4	15	—2	1.639	4453
4.4	20	—2	1.925	5518
4.6	15	—2	2.302	4728
4.6	20	—2	2.917	6615

TABLE VII.—PRECIPITATION OF ANTITOXIN (FRACTION 2) FROM EFFLUENT 1*b* AT —5° AND IONIC STRENGTH ABOUT 0.02

Fraction			
*p*H	EtOH, %	Yield, Gm.	Lf Units/Gm.
6.7	20	2.231	16,800
6.7	30	2.279	16,811
6.7	40	2.314	...
7.0	20	1.608	17,150
7.0	30	2.170	17,500
7.0	40	2.258	17,400
7.2	20	1.760	18,618
7.2	30	2.077	18,641
7.2	40	2.172	18,666
7.4	20	1.682	18,323
7.4	30	2.194	19,059
7.4	40	2.284	18,865

The conditions selected from Table VI for the precipitation of discard fraction 1*b* were: *p*H 4.4, ethanol 20%, temperature —2°, ionic strength 0.017. A large portion of plasma was processed to the effluent 1*b* stage and various equal aliquots were used to determine the proper conditions for precipitation of fraction 2 (digested antitoxin). The conditions of *p*H and alcohol concentration are given in Table VII. The temperature was maintained constant at —5°, and the ionic strength being relatively immaterial at this stage was uncontrolled but was within the region of 0.02. From the data in Tables V through VII, Method 3 was constructed which yields an enzyme digested alcohol fractionated antitoxin.

Statement of Method 3.—Add an equal volume of 2° water to antitoxic horse plasma. Adjust to about *p*H 6.0 with hydrochloric acid, add 0.36 Gm. 1:10,000 pepsin per 100 cc. of plasma, then adjust the *p*H to 4.0 and maintain at 2° for ninety-six hours. Precipitate the total globulin fraction at 35% ethanol, *p*H 6.6, —5°, and ionic strength 0.073. Collect the total globulin fraction by centrifugation at —5° and redissolve in 2° water, of *p*H 4.0, to the original plasma volume. Adjust the *p*H to 5.1, remove and discard the insoluble material by centrifugation at +2°. Adjust the effluent to *p*H 4.4, ethanol 20%, —2°; ionic strength 0.017. Remove and discard the insoluble material by centrifugation at —2°. Adjust the effluent to *p*H 7.4, ethanol 40% —5°, ionic strength about 0.02. Remove the antitoxic globulin by centrifugation at —5°. Suspend the precipitate in a minimal amount of ice water, freeze, and dry by vacuum from the frozen state.

In the case of antidiphtheria plasma of average potency, about 50% of the activity can be recovered with potency of approximately 20,000 Lf u./Gm. The dried material can be dissolved to the desired concentration in physiological saline or 0.3 molar glycine and can be sterilized by Seitz filtration.

DISCUSSION

Of the three methods described for the cold alcohol fractionation of equine antitoxic plasma, Method 1 gives a product of definitely higher yield and slightly higher potency. However, the resulting antitoxin is unmodified by the enzyme digestion which is customarily considered to be beneficial in reducing the incidence of "serum sickness." Whether or not this enzyme digestion is really beneficial must be decided by extensive clinical comparisons of digested and undigested antitoxins of equal purity.

Of the two methods applicable to pepsin digested equine antitoxic plasma, Method 2 affords slightly higher recoveries and slightly higher purities than does Method 3. Method 3 is preferred, however, because of the much smaller volumes required.

The methods commonly used for the purification of antitoxic globulin employ fractionation with salts at elevated temperatures and require a dialysis for the eventual removal of salt. Such methods are not applicable to large-scale processing, and the temperatures employed are favorable for the formation of pyrogen. The cold alcohol methods, on the contrary, allow the preparation of antitoxin of very low pyrogenicity.

SUMMARY

1. The cold alcohol fractionation of heat-defibrinated antidiphtheria equine plasma was investigated within the ranges of *p*H 6.2 to 7.5, ethanol 16 per cent to 25 per cent, temperature 0° to −5°, and at ionic strength of approximately 0.08.

2. The cold alcohol fractionation of unmodified antidiphtheria equine plasma was investigated within the ranges of *p*H 5.1 to 7.4, ethanol 8 per cent to 35 per cent, temperature −2° to −5°, and at ionic strength of approximately 0.05.

3. The changes occurring during pepsin digestion of equine antitoxic plasma were investigated within the ranges of *p*H 2.4 to 4.0, ionic strength 0.02 to 0.08, and at a temperature of approximately 2°. The customary modification of the plasma proteins by pepsin was not obtained under these conditions at an ionic strength of 0.02, but was obtained at an ionic strength of 0.08.

4. The cold alcohol fractionation of pepsin digested antidiphtheria equine plasma was investigated within the ranges of *p*H 4.2 to 7.4, ethanol 5 per cent to 40 per cent, temperature −1° to −5°, and ionic strength 0.015 to 0.07.

5. Two methods are presented for preparing antitoxin of satisfactory yield and potency. One is applicable to unmodified antitoxic plasma and the resulting antitoxin is composed of the three antitoxin bearing globulins (γ, T, and β). The other is applicable to pepsin digested plasma and the resulting antitoxin is composed of essentially a single electrophoretic component.

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The Disintegration of Compressed Tablets*

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A new type of apparatus for determining the disintegration rate of compressed tablets is described. Methods previously described are briefly reviewed. Data on results obtained by the new procedure are reported.

COMPRESSED tablets are one of the most popular forms of medication today, and both the United States Pharmacopœia and the National Formulary have admitted a variety of tablets as official preparations. The fact that the efficiency of a tablet is to a great extent influenced by the speed with which it disintegrates was recognized as early as 1902 (1), and in recent years both the British and the Swiss Pharmacopœias have included official methods for determining the time of disintegration for compressed tablets. However, in the United States, there is a variety of procedures currently in use for determining this factor, and there is a considerable discrepancy in the speeds with which different makes of the same type tablet disintegrate. All tablet manufacturers have disintegration tests which they apply to their tablets. According to a bulletin presented at a recent meeting of the American Pharmaceutical Manufacturers' Association, there are at least 12 different methods and types of apparatus currently in use for testing disintegration time (10).

The chief difficulty in determining disintegration time lies in the fact that different investigators interpret the word "disintegration" differently, frequently using solubility time for disintegration time; whereas in reality there should be a distinction between the two. In general, compressed tablets will do one of two things when put into a liquid: they will actually disintegrate, or fall apart, or they will slowly dissolve. The fact that a tablet disintegrates does not necessarily mean that it has dissolved; it is merely broken up into smaller particles. Consequently, in setting up standards for tablet disintegration, the fact that some types of tablets (usually those which do not contain a disintegrating agent) actually dissolve should be recognized.

The ideal definition of true disintegration time would be the time required for a tablet to break up into granules the size of the original granules from which it was compressed. However, manufacturing procedures vary so much that it would be difficult to establish a common size granule for all tablets; so in this investigation the granules in the above definition were considered to be of a size small enough to pass through an 8-mesh screen, since the majority of tablet granulations do not exceed that size.

This investigation was prompted by a desire to compare the most popular procedures now in use, and to discover, if possible, a simple method of testing disintegration time which would give consistent results with a minimum amount of equipment. If a standard method for determining the disintegration time of tablets could be accepted as satisfactory by all manufacturers, definite requirements could then be established for the official tablets, thereby insuring a uniformity of time of action as well as type of action.

Considering the fact that the principal testers of tablets are the industrial houses, those methods which by virtue of simplicity and/or compactness seemed most acceptable to use in an industrial laboratory were chosen for comparative tests. A brief description of these methods follows.

METHODS

The Industrial Method.—Various industrial firms who were contacted reported that they used with slight modifications the same method, which is known as the beaker and screen method. This consists simply of a wire screen of appropriate size supported in a beaker of water. The tablet to be tested is dropped onto the screen, which is then gently agitated. The time of disintegration is taken as the time required for the tablet to break apart and fall through the screen.

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The Modified Berry Method.—This type of apparatus consists of a beaker, over the top of which is a platform with a narrow opening in it. Through the opening a thread loop is placed over the tablet to be tested and on the end of the loop is a 50-Gm. weight. The beaker is filled with water and the tablet is allowed to rest over the opening in the platform, with the weight supported by it. The disintegration time is taken as the time required for the weight to break through the tablet and fall to the bottom of the beaker (5).

The A. O. A. C. Method.—Similar to the industrial method, this type consists of a wire gauze suspended in a beaker of water which also has an electric stirrer in it. The tablet is dropped into the gauze below the surface of the water, which is continuously agitated by the stirrer. The disintegration time is measured as the time required for the tablet to break apart and fall through the screen (6).

The Lewis Method.—This is a simple apparatus consisting of a piece of muslin stretched over the top of a small funnel, drum fashion, on top of which rests a flat spiral of wire about $^1/_{32}$ inch thick, on which the tablet is placed. At a height of 6 inches above the spiral is a jet from which one large drop of solvent falls on the tablet every second. The disintegration time is taken as the time required for the tablet to completely fall apart (2)

The É'we Method.—The principle of a glass tube of about 30 mm. inside diameter, the bottom of which is covered by a wire screen with a shallow depression in the center is used for the É'we Method. This tube is immersed in a beaker of fluid, the tablet is dropped into the tube, and a hog-bristle paint brush weighing about 30 Gm. is introduced into the tube so that the bristles rest on the tablet; then the brush is gently raised and lowered at the rate of 15 times per minute "until the tablet is dissolved or until it is disintegrated as shown by the breaking of the last portion of the tablet into at least several pieces" (3).

The Gershberg and Stoll Method.—This type of apparatus, which Gershberg and Stoll report as having been used by the laboratories of the U. S. Army Medical Department since 1940, basically consists of a rack containing glass tubes whose bottom openings are covered with wire screen. This rack is immersed in a beaker of water and a tablet is put in each tube. The rack is raised and lowered in the water at the rate of about 20 times per minute, and the disintegration time is taken as the time required for the tablets to break apart and fall completely through the screen (8).

The A. Ph. A. Method.—Proposed by Berry and modified by the A. PH. A. laboratory, this apparatus requires five test tubes which can be stoppered tightly and attached to a wheel or shaft which can be turned. This wheel is immersed in a constant temperature water bath, the tubes being filled with water, and a tablet is put into each tube. The tubes are stoppered and the wheel revolved slowly so that the tablets move gently from one end of the test tube to the other. The time required for the tablet to fall apart is taken as the disintegration time (7).

EXPERIMENTAL

Samples of competitive type tablets were obtained from seven different sources. Ten tablets from each lot were tested by each of the above methods, the time required for disintegration being checked with a stop watch. In order to standardize conditions, distilled water at room temperature was used as the liquid in all cases, and wherever a screen was required, a standard 8-mesh wire screen was employed. The average time required for disintegration or solubility of the ten tablets was calculated for each method, and is shown in Table I.

From the results presented in Table I, it can be seen that a considerable variation of disintegration time is obtained for the same tablet, using different methods; and that the greatest variation is in those tablets which have a tendency to dissolve rather than disintegrate, such as sodium salicylate and methenamine.

DISADVANTAGES OF METHODS TESTED

Each method tested was found to have certain disadvantages, which may be briefly summarized as follows:

The Industrial Method, while satisfactory for tablets which disintegrate quickly, is inconvenient for those which require a long time to disintegrate, and it is difficult for different operators to maintain the same speed of agitation of the screen. Also, this method is not practical for certain tablets which have a tendency to float on the surface of he liquid.

The Modified Berry Method, which is more of a stress test than one for disintegration, does not give a true disintegration time, since the suspended weight usually breaks the tablet in half; and certain tablets with transverse laminations resist the wire force since the weight does not permit them to swell properly and disintegrate normally. In many cases, these tablets contain hard cores which resist disintegration but do break under the pressure of the weight to give a false end point.

The A. O. A. C. Method has one chief drawback in that the current induced by the stirrer in the water has a tendency to draw the tablet up against the moving stirrer, which then breaks it up mechanically to give a false disintegration.

The Lewis Method is too time-consuming, since only one tablet can be tested at a time; and the force of the dropping water tends to push the tablet out of the path of the water so that it is no longer in the center of the dropping liquid.

The É'we Method, in our opinion, causes a false disintegration due to the unavoidable contact of the eccentric-geared brush with the tablet, which tends to push it through the screen.

The Gershberg-Stoll apparatus is cumbersome and makes an exact end point difficult to determine

TABLE I.—DISINTEGRATION TIME IN SECONDS

Company	Tablet	Industrial	Lewis	A. O. A. C.	Berry	Gershberg–Stoll	É'we	A. PH. A.
A	Aspirin, 5 gr.	26	29	19	18	26	29.1	22
	P. A. C.	46	51.3	48	45	54 3	61	52.4
	Sod. Bicarb., 5 gr.	15	26.8	19	5.5	12.7	13	28
	Atropine Comp.	132	129	135	75	131	135	160
	Ascorbic Acid.	570	815	900	[a]	791	1680	850.3
B	Aspirin, 5 gr.	19.6	18.9	19.6	17.9	17.1	21.7	14.3
	Sulfasuxidine	26	28.3	27	21	22.1	41.4	28.2
	Sod. Salicylate, 10 gr.	1320	[a]	[a]	[a]	[a]	[a]	[a]
	Phenobarb., $1^1/_2$ gr.	15	10.9	15.2	9.2	7.8	11	8.6
C	Aspirin, 5 gr.	15.5	12.2	16	12.1	14	20	10.4
	Sulfathiazole	84	93	131	79.8	100	102	95
	Chloramine-T	210	190	504	588	390	651	365
	Ephedrine Comp.	635	643	598	705	785	546	888
	Sod. Salicylate, 5 gr.	390	540	[a]	960	858	1416	1280
	$KMnO_4$, 5 gr.	16	10	8	3.8	8.4	7	?
D	Aspirin, 5 gr.	7.6	14	40.3	12.8	15	9.5	16
	P. A. C.	186	274	383	518	285	397	479
	Thiamin HCl	216	260	378	760	370	402	354
	Sod. Bicarb., 5 gr.	54.9	49	69	110.6	85	110.6	84
	Ascorbic Acid	234	292	508	630	519	1202	572
	Sod. Salicylate, 5 gr.	450	481	585	782	844	965	672.5
E	Aspirin, 5 gr.	14.6	14	15.6	13.2	11.3	15.1	13
	Methenamine, 5 gr.	258	210	367	377.5	194	535	375
	Ca. Lactate, 5 gr.	240	270	284	214.8	156	271	270
	Sod. Bromide, 5 gr.	130	106	137	82.4	132	130	140
	P. A. C.	47.5	40	70	[a]	185	[a]	[a]
	P. A. C. Yellow	54.1	110	94	[a]	[a]	[a]	[a]
F	Aspirin, 5 gr.	5	11.2	4.8	5	7	7.1	6.9
	P. A. C.	9.6	18.4	14.1	8.4	11.5	18 7	28.8
	Acetanilid, 5 gr.	53.9	36.2	65	37.4	45.6	130.8	43.2
	Phenobarb., $1^1/_2$ gr.	133	112	111	135	105	137.4	113
	Bismuth Comp.	27	51.7	44	19.4	28	35	46
G	Aspirin, 5 gr.	10	9.8	29	8.9	17	11.6	15.1
	Sulfathiazole	104	110	105	68	119	122	94
	Phenacetin	43.2	37	105	45.4	51.2	32.5	70
	Mg. Trisilicate	33.6	25.7	79	17	26.2	29	29
	Ca. Lactate	25.1	38.4	26.8	5.4	12	22.8	35.5
	Ca. Carbonate	409	630	[a]	310	620	498	[a]
	Ascorbic Acid	60	101	86.6	86.6	58	96.3	115
	Thiamin	159	178	309	145	196	220	205
	Bi. Subgallate	135	94.9	176.2	69.6	110	101.8	130

[a] Tablets not completely disintegrated after thirty minutes.

because the disintegrating tablets tend to cloud up the solution which is churned by the up-and-down motion of the basket. Also, since the tubes are arranged in a circular rack, it is difficult to see them all clearly at the same time.

The A. PH. A. Method has two main objections: Too much time is required in getting the tablets in the tubes, stoppering them, and starting them in motion, and also in many cases turbid solutions are produced which make it difficult to see the end point.

Also, in the last three methods, the size of the tubes is such that many larger tablets will not fit into them and thus cannot be tested.

APPARATUS

Considering all factors involved in determining tablet disintegration, the ideal apparatus should, above all, be of simple construction so that standardized apparatus can be easily put together; it should be of such a nature that the tablets are continuously immersed in the testing liquid; the tablets should be kept in continuous gentle motion without being subjected to any unnatural stress; a definite means of establishing an end point should be specified; and the liquid in which the test is run should be kept clear so that the exact end point can be determined. With these requirements in mind, the following apparatus was designed as suitable for determining the disintegration time of tablets:

A circular cylinder $1^1/_2$ inches in diameter and $2^1/_2$ inches long was made from 8-mesh wire gauze. Each end of the cylinder was closed with 8-mesh wire disks, to the centers of which were soldered small axles, with a pulley wheel of the same diameter as the cylinder attached to one of the axles. A small trap door which could be opened and closed was made in the side of the cylinder, which was then suspended by the axles between two aluminum supports which had been built around a 1000-cc. beaker, so that the cylinder revolved freely on the axles. The pulley wheel was connected by a belt to another pulley attached to a small motor on a ring stand

TABLE II —COMPARATIVE DISINTEGRATION TIME OF CERTAIN TABLETS

Company	Tablet	DeKay-Evanson-Sperandio Method Disintegration	Av. of Other Methods, Sec.
A	Aspirin, 5 gr.	26.2	24.1
	P. A. C.	56.5	51.1
	Sod. Bicarb., 5 gr.	18.7	17.1
	Atropine Comp.	127	128.1
B	Aspirin, 5 gr.	19	18 4
	Sulfasuxidine	28.5	27.7
	Phenobarbital, 1½ gr.	11	11.1
C	Aspirin, 5 gr.	13.8	14 3
	Sulfathiazole	114	97.8
	Chloramine-T	452	412.5
	Ephedrine Comp.	690	671.4
	Sod. Salicylate, 5 gr.	763	777.7
D	Aspirin, 5 gr.	14.4	16 4
	P. A. C.	394	360.3
	Thiamin, 50 mg.	385	391.4
	Sod. Bicarb., 5 gr.	84	80 4
	Ascorbic Acid	474	565.2
	Sod. Salicylate, 5 gr.	651	667
E	Aspirin, 5 gr.	12.7	13.8
	Methenamine, 5 gr.	307	330.9
	Ca Lactate, 5 gr.	266	243 7
	Sod. Bromide, 5 gr	118	122 5
F	Aspirin, 5 gr.	6.3	6.7
	P A. C.	14.9	19.5
	Acetanilid, 5 gr.	69	68.8
	Phenobarbital, 1½ gr.	134	126.6
	Bismuth Comp.	30	35.8
G	Aspirin, 5 gr.	14	14.5
	Sulfathiazole	88	103 2
	Phenacetin	186.1	54.9
	Mag. Trisilicate	40	34.2
	Ca. Lactate	33	23.7
	Ca. Carbonate, 10 gr.	1105	493
	Ascorbic Acid	96	86.2
	Thiamin	204	201
	Bi-Subgallate	105	116.9

directly above the beaker. The beaker was then filled with distilled water at room temperature to a depth just sufficient to cover the top of the wire basket. To maintain a clear solution, an outlet tube was blown into the bottom of the beaker and an inlet tube was introduced into the top. Distilled water was then run in at the top and drained out at the bottom at a rate of 100 cc. per minute during the disintegration tests. The tablet or tablets were dropped into the trap door, which was then closed and the motor adjusted so that the basket rotated in the water at the rate of ten complete revolutions per minute. The apparatus is illustrated in Fig. 1. The time of disintegration was taken as the time required for the tablets to break apart into particles which would fall through the basket to the bottom of the beaker.

Each lot of tablets which had been tested by the preceding seven methods was then tested by the DeKay-Evanson-Sperandio Method and the average disintegration time for ten tablets from each lot was taken. For comparative purposes, this disintegration time is shown with the *average* disintegration time of the other seven methods combined. Table II.

It can be seen from Table II that the proposed method gives a disintegration time quite close to the average time of all the other methods tried in almost every case. There is a considerable variation in the case of the ascorbic acid tablets of Company D, and a very great difference in the case of the Phenacetin Tablets and Calcium Carbonate Tablets of Company G. It was found that in these cases where the disintegration time was much longer than average, it was because the granules into which the tablet broke up were of a larger size than the 8-mesh screen, and consequently had to disintegrate further before reaching the established end point.

As a further test for the efficiency of the DeKay-Evanson-Sperandio Method, it was decided to determine the disintegration time of the various samples of aspirin tablets, testing them one at a time and in increasing amounts up to five at a time, to see if consistent results could be obtained. These results are shown in Table III.

From this tabulation, it would seem that from one to five tablets can be tested at one time, depending on the wish of the operator, and that repeatable results can be obtained.

All tablets do not disintegrate at the same time given for a particular lot. Even though they may be stamped on the same machine, at the same time, at the same pressure, and from the same granulation, tablets will deviate from the average disintegration time. Table IV shows such deviation for a representative lot of tablets from various manufacturers. Since data for other methods can be found elsewhere, only the DeKay-Evanson-Sperandio Method will be considered here

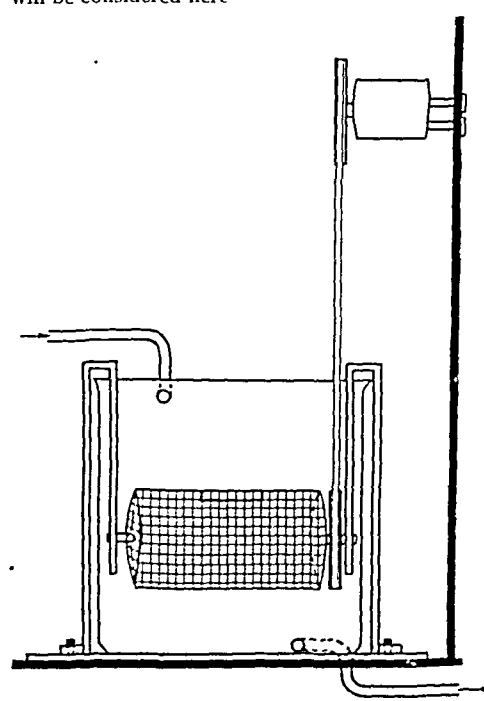

Fig. 1.—DeKay-Evanson-Sperandio Apparatus

TABLE III.—COMPARATIVE DISINTEGRATION TIME OF ASPIRIN TABLETS

No. of Tablets Tested at One Time	Disintegration Time, Sec.						
	Co. A	Co. B	Co. C	Co. D	Co. E	Co. F	Co. G
1	24	12	14	11	13	7	13.5
2	26.6	18	14.5	13	12.5	8	14
3	26	18	16	13.5	13	7	10.6
4	28	21	15.2	15	13.5	7.2	11.5
5	25.2	20	16.5	14	14	6.5	10
Av.	25.9	17.8	15.2	13.5	13.2	7.1	11.9

TABLE IV.—DISINTEGRATION TIME DEVIATION FOR A REPRESENTATIVE LOT OF TABLETS

Co.	Tablet	Disintegration Time, Sec.										Av.	Deviation, Sec.
B	Phenobarbital, 0.5 gr.	13	17	12	11	16	11	11	12	14	11	12.8	+4.2 −1.8
C	Sulfathiazole, 7.7 gr.	140	126	125	128	125	135	125	137	130	126	129.6	+10.4 −4.6
	Chloramine-T, 4.6 gr.	508	513	492	532	520	457	460	475	514	529	500	+32.0 −43.0
D	P. A. C.	439	306	313	408	345	339	422	430	365	355	372.2	+66.8 −66.2
	Sod. Bicarb., 5 gr.	72	114	88	45	148	122	51	58	55	57	80.8	+67.2 −35.8
	Thiamin HCl, 50 mg.	397	384	375	375	387	375	404	366	410	358	383.1	+26.9 −25 1
F	Aspirin Comp.	20	16	16	15	20	20	18	18	20	19	18.2	+1.8 −3 2
	Bismuth Subcarb.	33	36	33	32	33	35	31	33	34	33	33.3	+2 7 −2.3
	Phenobarbital, 1.5 gr.	117	189	115	203	180	135	190	191	190	175	132.5	+70 5 −17.5
G	Ascorbic Acid, 25 mg.	72	91	93	86	93	114	96	98	91	93	92.7	+21.3 −20 7
	Calcium Lactate 5 gr.	26.5	33	30.5	30	30.5	31	33	27	32	30	30.3	+2 7 −3 2
	Thiamin HCl, 5 mg.	260	292	263	270	252	245	273	251	241	247	259.4	+32 8 −14 4

SUMMARY AND CONCLUSIONS

Disintegration tests were run on a total of over 4000 tablets from seven different manufacturers, using seven different methods for determining the disintegration time.

It was found that no two methods consistently give the same average disintegration time for the same batch of tablets, and that some methods are not satisfactory for all types of tablets.

Several of the disintegration methods tried were deemed unsatisfactory because: (*a*) they did not have a definite end point which could be interpreted the same by all operators; (*b*) they produced a false end point either through stress or pressure on the tablet; (*c*) the end point was difficult to determine due to cloudiness or coloring of the solution.

A new method for determining disintegration time was devised and the various tablets were tested by it. The results obtained were found to compare favorably with the averages of the other methods combined.

We recommend this new method—the DeKay–Evanson–Sperandio Method—as an improved means of determining an accurate disintegration time for all types of tablets for the following reasons:

It gives a true disintegration time. The disintegration of the tablet is neither impeded nor accelerated by any physical force or unnatural shaking.

The end point is always definite and is the same for all tablets. The solution is kept clear, allowing the end point to be seen.

The disintegration times obtained are consistent for the same types of tablets.

From one to five tablets can be tested at one time.

All types of tablets can be tested.

It is simple to construct and occupies very little space.

Due to the many variable factors which influence the disintegration of compressed tablets, it would be necessary to make exhaustive tests and comparisons of all the accepted methods before one method could positively be established as preferable to all others; but it is inevitable that at some future time, a standardized method will be accepted officially. When this is done it will aid greatly in the production of uniform U. S. P. and N. F. tablets.

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A Study of the Physical and Chemical Properties of Natural Washington and Oregon Peppermint Oils*

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This report covers a four-year study of peppermint oil from various districts of Washington and Oregon. The following physical and chemical properties were ascertained for oils from each of the districts: total menthol, esters, optical rotation, specific gravity, refractive index, and solubility.

THE production of peppermint oil in the United States had its inception in 1816 in Wayne County, New York. In 1835 the cultivation of peppermint was introduced into Michigan and in a short period of time the industry spread into Indiana About 1919 peppermint cultivation was initiated on the Pacific Coast and today this enterprise has developed into a large and flourishing industry. The abundance of suitable land, temperate climate, and an ample water supply are all contributing factors to the phenomenal growth of the peppermint industry in the states of Washington and Oregon.

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Main Oil-Producing Districts of Washington and Oregon

This study concerns four distinct types of peppermint oils, *viz.*, C, E, S, and K, depending upon the particular district in which the peppermint is grown. Following is a brief description of each district.

Type C.—The district producing this oil lies along the Washington and Oregon sides of the Columbia River approximately 40 miles from the ocean. The land has been diked and is drained by a series of ditches. Subirrigation is carried on during the growing season when necessary by raising the water level in the ditches. The soil is a combination of peat, river silt, clay loam, and black loam. The rainfall in this district is about 40 inches per year.

Type E.—This oil is produced along the upper Willamette and lower McKenzie rivers in the state of Oregon. The Willamette Valley at this point has an average rainfall of about 35 inches per year and the soil is a deep, sandy river silt. Water from creeks and small rivers is used in irrigating the peppermint by means of overhead sprinklers.

Type S.—The oil is obtained from peppermint grown on river silt and clay loam soil in the central section of the Willamette Valley in the state of Oregon. The average annual rainfall is about 33 inches and the mint land is irrigated by overhead sprinkler systems using water obtained from shallow wells.

Type K.—This oil is produced in eastern Washington, a dry region with an average annual rainfall of less than 6 inches. The soil is a rich, deep lava ash and requires irrigation.

EXPERIMENTAL

The lack of significant analytical data regarding the physical and chemical properties of natural peppermint oil produced in the four districts of Washington and Oregon previously mentioned in this paper provided a need for this study. Since the oils from the various localities are distinct in their odor and flavor, it was reasonable to assume that some of their physical and chemical constants would vary.

In most cases each sample is representative of a full drum (400 pounds) of oil and all the samples used in this investigation were from commercial lots of oil. To the best of our knowledge the age of these samples at the time of analysis varied from one to six months, the greater part were not over three months old when analyzed.

Constants and Methods Used

Esters.—The U. S. P. XII method was used and the esters were calculated as menthyl acetate.

Total Menthol.—The U. S. P. XII procedure and formula for calculating the per cent of menthol were employed.

Optical Rotation.—The optical rotation was obtained at 25° using a 100-mm. tube and a Rudolph routine polarimeter equipped with a General Electric sodium laboratory arc as the source of light.

Specific Gravity.—A 10-Gm. pycnometer with attached thermometer was used in determining the specific gravity (25/25°).

Refractive Index.—This constant was ascertained with an Abbe refractometer at 20°.

Solubility.—The procedure designated in the U. S. P. XII for dementholized or impure peppermint oil was used. A soluble oil was rated as one clearly soluble at 25° in 3 volumes of 70% alcohol, by volume, with no separation of oil globules.

RESULTS

The values obtained for the various physical and chemical constants of natural Washington and Oregon peppermint oils are presented in tabular form. The minimum, maximum, and average results are presented in Table I for each of the four types, together with the year the oil was produced and the number of samples analyzed.

Table II shows the solubility data of the several types of natural peppermint oils in 70% alcohol at 25°. As designated in the United States Pharmacopœia XIII, the soluble oils are clearly soluble in 3 volumes of 70% alcohol, by volume, with no separation of oil globules.

TABLE I.—SOME PHYSICOCHEMICAL CONSTANTS OF NATURAL WASHINGTON AND OREGON PEPPERMINT OILS

No. of Samples	Year	Type of Oil		Esters, %	Total Menthol, %	Optical Rotation, 100-Mm./25°	Specific Gravity, 25°/25°	Refractive Index, 20°
33	1942	C	Min.	4.0	43.9	−21.0	0.8974	1.4622
			Max.	7.3	61.2	−26.3	0 9032	1 4663
			Av.	5.1	52.5	−23.9	0 9025	1.4633
146	1943	C	Min.	2.9	42.5	−17.0	0.8966	1.4606
			Max.	8.7	64.3	−31.0	0.9161	1 4675
			Av.	4.8	51.9	−23 4	0.9007	1.4624
27	1943	E	Min.	3.6	47.0	−20.0	0.8969	1.4610
			Max	11.2	66.2	−27.0	0 9131	1 4648
			Av.	6.4	58.3	−23 2	0.9025	1 4635
104	1943	K	Min.	3.0	38.9	−7.0	0.8812	1 4630
			Max.	9.9	64.0	−22.2	0.9190	1.4680
			Av.	5.7	53.2	−16.3	0.9144	1 4655
57	1944	C	Min.	3.53	45.64	−18.0	0 8995	1 4613
			Max.	11.27	66.44	−29.2	0.9141	1 4672
			Av.	5.90	55.65	−22.7	0 9031	1.4632
10	1944	S	Min.	4.07	49.07	−18.0	0.9004	1.4620
			Max.	9.66	62.19	−26.8	0.9050	1.4653
			Av.	7.36	57.43	−22.6	0 9032	1.4636
213	1944	K	Min.	3.60	36.63	−9.0	0.9017	1.4618
			Max.	16.26	72.07	−22.5	0.9519	1.4712
			Av.	6.36	52.47	−15.2	0 9084	1 4653
101	1945	C	Min.	4.12	42.87	−15.5	0.8993	1.4612
			Max.	12.00	67.06	−29.0	0.9135	1.4670
			Av.	6.74	56.25	−24.1	0.9024	1.4632
108	1945	E	Min.	5.03	52.23	−19.8	0.8979	1.4610
			Max.	14.80	69.91	−29.7	0.9078	1.4647
			Av.	7.86	59.44	−25.1	0.9019	1.4618
58	1945	S	Min.	4.50	47.20	−17.7	0.8998	1.4618
			Max.	11.50	64.99	−27.4	0.9286	1.4681
			Av.	7.36	56.98	−23.1	0.9032	1.4632
202	1945	K	Min.	3.94	39.15	−10.4	0.8990	1.4622
			Max.	10.96	65.21	−26.0	0.9271	1.4672
			Av.	6.65	53.60	−18.5	0.9057	1.4646

DISCUSSION

Tabulated below are the U. S. P. standards of peppermint oil and the average results of each type of natural oil for the years in which the investigations were made as designated in Table I above.

In Table I the maximum and minimum figures listed represent extreme values for the various constants investigated and are not representative of the true value of the various oils. Therefore for comparison with the U. S. P. standards only the average figures are considered. From the above table it is apparent that the average ester and menthol content of the four types of natural peppermint oils meet the U. S. P. standards. Likewise the optical rotation, specific gravity, and refractive index of types C, E, and S natural oils conform to the pharmacopœial requirements. However type K with an average rotation of −16.7 (U. S. P. −18 to −32) is lower than the official standard. Furthermore the specific gravity of type K, 0.896 (U. S. P. 0.896 to 0.908) and refractive index, 1.4651 (U. S. P. 1.4590 to 1.4650) are slightly higher than the official limits. One must bear in mind that all of these values are based on natural oils and under normal rectification there is an alteration to a certain extent of the constants in redistilled oils.

Preliminary studies indicate that the specific gravity and refractive index of type K peppermint oil will fall within the U. S. P. limits upon rectification. However, the optical rotation is not sufficiently altered by this treatment to conform to the standards of the Pharmacopœia.

The investigation of Washington and Oregon peppermint oils is being continued in the Pharmaceutical Chemistry Laboratories at the University of Washington.

TABLE II.—SOLUBILITY OF NATURAL WASHINGTON AND OREGON PEPPERMINT OILS IN 70% ALCOHOL

Type of Oil	No. of Samples	Soluble, %	Insoluble, %
C	337	95.41	4.59
E	135	97.04	2.96
S	68	98.55	1.45
K	519	85.00	15.00

TABLE III.—COMPARISON OF THE AVERAGE PHYSICOCHEMICAL CONSTANTS OF THE VARIOUS TYPES OF NATURAL PEPPERMINT OILS WITH THE STANDARDS OF U. S. P. XIII PEPPERMINT OIL

Type of Oil	No. of Samples	Ester, %	Total Menthol	Optical Rotation, 100 Mm./25°	Specific Gravity 25°/25°	Refractive Index, 20°
C	337	5.60	53 90	−23.5	0.9018	1.4629
E	135	7.57	59 21	−24.7	0 9020	1.4621
S	68	7 36	57 05	−23 03	0.9032	1.4633
K	519	6.34	53.06	−16.7	0.9086	1.4651
U. S. P.	...	Not less than 5	Not less than 50	−18 to −32	0 896 to 0.908	1.4590 to 1.4650

SUMMARY

1. The results of a study on the physical and chemical constants of natural peppermint oil from four districts of Washington and Oregon are reported.

2. A study of the solubility characteristics of these four oils is included.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

1,1,1-Trichloro-2,2-bis(*p*-aminophenyl)ethane
3-Geranylcatechol
Catechol monophenyl ether
5-Vinylguaiacol
5-Allylguaiacol
3-Methyl-4-thioquinazolone-1,3
Maltobionic acid
2-Propenylpyridine
1-Tartaric acid
Embden ester
Chlorogenine
Digitogenine
Benzoxanthene
17-Hydroxycorticosterone
11-Dehydro-17-hydroxycorticosterone
17-Hydroxy-11-desoxycorticosterone
Pregnene-4-triol-17(β),20(β),21-dione-3,11-monoacetate-21
Pregnene-4-triol-17(β),20(β),21-dione-3,11-diacetate-20,21
Pregnene-4-diol-17,20-one-3-al-21

Adjusted Collyria Containing Zinc Salts*

By WILLIAM J. HUSA† and JACK K. DALE‡

Experiments on complex compounds of zinc led to the development of zinc collyria from which the zinc is not precipitated at *p*H 7.4, which is the approximate *p*H value of the tear secretion. It was found that citrates, tartrates, and glycine may be added to borate buffers to produce stable zinc collyria with *p*H values as high as 8.7. A new barbital-sodium barbital buffer has been devised which has maximum buffer capacity at the approximate *p*H of the tear secretion.

DILUTE solutions of zinc salts, with or without other medicaments, are widely used as ophthalmic astringents. To prevent precipitation of the zinc by the alkalinity of the tear secretion, it is common practice to make the solution acid by use of boric acid or acid buffer solutions.

The purpose of the present investigation was to develop collyria from which the zinc is not precipitated at *p*H 7.4, which is the approximate *p*H value of the tear secretion. This work involved a further study of buffers and led to the preparation of a new barbital-sodium barbital buffer which has maximum buffer capacity at the approximate *p*H of the tear secretion.

Collyria containing zinc have been used since ancient times. A zinc sulfate collyrium was included in an American military pharmacopœia in 1781 (1). The U. S. P. of 1820 contained formulas for collyria of zinc acetate and zinc sulfate (2). Various articles concerning precipitates in solutions of zinc salts appeared during the nineteenth century; these precipitates were usually removed by filtration. One of the first attempts to insure solution of all the zinc is found in the formula for zinc sulfate solution of the Edinburgh pharmacopœia, in which a small proportion of dilute sulfuric acid was used (3).

The problem of preparing zinc collyria free of precipitated basic salts was not very successfully solved until 1918, when Peacock and Peacock (4) showed that precipitation in zinc chloride collyria could be prevented by use of boric acid and ammonium chloride.

H. Lipschutz (5) in 1929 demonstrated that the alkalinity of the tears was sufficient to precipitate all the zinc from an unbuffered 0.33% zinc sulfate collyrium. He first recommended an acetate buffer of *p*H 5.17 but later suggested the use of 0.5% to 1% of ammonium chloride.

Weakly buffered boric acid solutions and borate buffers with *p*H values from 4.2 to 6 have been recommended as suitable vehicles for zinc salts (6–9).

An isotonic zinc collyrium buffered and stabilized with boric acid and sodium acetate at *p*H 6.75 was introduced by Arrigoni, Fischer, and Tozer in 1941 (10); this collyrium contained methyl and propyl *p*-hydroxybenzoates as preservatives against molds and fungi.

Bijleveld and Dekking (11) in 1941 found that the addition of sodium citrate permitted the *p*H of borate buffered zinc collyria to be raised to 6.2 or higher without precipitation. In the same year Vogelenzang (12) published a formula for a similarly stabilized zinc sulfate collyrium having a *p*H of 7.2–7.4 and a freezing point of −0.80°.

EXPERIMENTAL

Barbital-Sodium Barbital Buffer Mixtures.—The practical usefulness of a weak acid in the preparation of a graduated series of buffer solutions is limited to a range of about 4 *p*H units. In general, the best buffer capacity is obtained when the concentrations of the acid and salt are approximately equal. In solutions containing equimolar concentrations of a weak acid and its salt, the negative logarithm of the ionization constant of the acid becomes numerically equal to the *p*H, i.e., $pK_a = pH$ under these conditions. Since the pK_a of boric acid is 9.24, the useful range of borate buffers is from about 7.24 to 11.24. Since borate buffers are the basis of Gifford's and Feldman's buffer solutions, it follows that these solutions are somewhat lacking in buffer capacity in the range of *p*H 6.6–7.8. Hence an effort has been

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made in the present investigation to develop a buffer system having better buffer capacity in the range *p*H 6.6–7.8 which is needed in ophthalmic practice.

A search was made for other chemicals with maximum buffer capacity in the vicinity of *p*H 7.4. Phosphate buffers with a pK_{a2} of 7.21 adequately cover the desired *p*H range but they are incompatible with many ophthalmic prescription ingredients, notably zinc and silver salts. Theoretically *p*-hydroxybenzoic acid should have maximum buffer capacity at *p*H 6.94 but this compound is too insoluble for practical use. Diethylbarbituric acid (Barbital, U. S. P.) has a pK_a value of 7.43 (13) and appeared promising although the slight solubility necessitates the use of 0.02 *M* solutions which are about one-tenth as concentrated as the usual borate buffers and hence have less buffer capacity.

A mixture of equal volumes of 0.02 *M* barbital, U. S. P., and 0.02 *M* sodium barbital, U. S. P., was prepared in order to compare the pK_a value with the actual *p*H value. The latter was found to be *p*H 7.93 at 26° as compared with the usually accepted pK_a value of 7.43. A *p*H value of 7.95 at 18° was reported by Britton and Robinson (14).

All *p*H determinations were made with the Beckman glass electrode *p*H meter, laboratory model G, using 5-inch external type glass electrodes. The Beckman alkali resistant glass electrode was used for determinations above *p*H 9. The *p*H meter was set at the temperature of the solution to be measured. Before use and after each ten *p*H determinations the *p*H meter was calibrated, using a 0.05 *M* potassium acid phthalate solution prepared from a standard salt supplied by the National Bureau of Standards. This solution has a *p*H of 4.01 at 25°.

The results of *p*H determinations on various barbital buffer mixtures are given in Table I.

The results in Table I indicate that the addition of 1% of sodium chloride lowered the *p*H of the barbital buffers. The addition of sodium citrate lowered the *p*H above 7.3 and increased the *p*H below 7.3. This effect illustrates the principle that neutralization of a mixture of two weak acids having *p*H values within several units of each other gives a *p*H curve which is approximately a straight line over the buffer range of both acids.

It is generally accepted that the activity of the hydrogen ion increases with increasing temperature, i.e. the *p*H of a solution drops as the temperature is raised. The results in Table I indicate that for modified buffer C, a temperature rise of one degree lowers the *p*H an average of 0.03 *p*H unit over the range 27° to 37°. Modified buffer C appears suitable for use over the *p*H range of 6.6 to 7.8 which is desired in ophthalmic practice.

TABLE I.—*p*H VALUES OF BARBITAL-SODIUM BARBITAL BUFFER MIXTURES

Buffer Mixture			*p*H Values			
Cc. 0.02 *M* Barbital	Cc. 0.02 *M* Sodium Barbital	Plain Buffer at 27°	Modified Buffer A[a] at 27°	Modified Buffer B[b] at 27°	Modified Buffer C[c] at 27°	Modified Buffer C[c] at 37°
0	10	9.76	9.55	9.39	9.52	9.04
1	9	8.83	8.65	8.71	8.69	8.35
2	8	8.50	8.33	8.40	8.38	8.02
3	7	8.27	8.11	8.19	8.18	7.82
4	6	8.06	7.92	8.00	7.98	7.63
5	5	7.91	7.76	7.83	7.80	7.48
6	4	7.73	7.59	7.66	7.61	7.30
7	3	7.55	7.39	7.49	7.42	7.12
8	2	7.33	7.15	7.30	7.22	6.92
9	1	6.99	6.80	7.09	6.95	6.70
10	0	5.13	5.01	6.83	6.60	6.41

[a] Plain buffer + 1% sodium chloride.
[b] Plain buffer + 0.5% sodium citrate.
[c] Plain buffer + 1% sodium chloride and 0.5% sodium citrate

TABLE II.—PROPORTIONS OF ACID AND ALKALINE BUFFERS REQUIRED IN PREPARING MODIFIED BUFFER C

Desired *p*H Value	Cc. Acid Buffer Solution[a]	Cc. Alkaline Buffer Solution[b]
6.6	9.3	0.7
6.7	9.0	1.0
6.8	8.6	1.4
6.9	8.1	1.9
7.0	7.7	2.3
7.1	7.2	2.8
7.2	6.6	3.4
7.3	6.0	4.0
7.4	5.5	4.5
7.5	4.9	5.1
7.6	4.2	5.8
7.7	3.6	6.4
7.8	3.1	6.9

[a] 0.02 *M* barbital solution containing 1% of sodium chloride and 0.5% of sodium citrate.
[b] 0.02 *M* sodium barbital solution containing 1% of sodium chloride and 0.5% of sodium citrate.

The quantities of acid and alkaline buffers necessary to give *p*H values from 6.6 to 7.8 at 37°, with intervals of 0.1 *p*H unit, were determined by interpolation of a graph based on the first and last columns of Table I. The *p*H values were checked experimentally and agreed with the interpolated values within 0.02 *p*H unit. The results are given in Table II.

Effect of Zinc Salts in Buffer Mixtures.—It is known that the *p*H at which a metallic hydroxide precipitates depends to some extent on the anions present. Hence different zinc salts were used in

Gifford's (8) and Feldman's (9) buffer solutions to determine which salts would give the least precipitation. Solutions of zinc acetate, bromide, chloride, iodide, nitrate, phenolsulfonate, and sulfate were prepared in 0.01 *M* concentration. Each of these solutions was mixed with an equal volume of a series of double-strength Gifford's buffer mixtures. Identical experiments were carried out with double-strength Feldman's buffer mixtures. The resulting mixtures contained zinc salts in 0.005 *M* concentration in buffer solutions covering the *p*H range of 5 to 7.4 with intervals of approximately 0.2 *p*H unit. All of the zinc salts gave clear solutions with both buffer systems up to and including *p*H 6.8, except for the bromide and chloride, which gave precipitates of basic salts over the entire range. Slight precipitates appeared at *p*H 6.99 with the acetate and sulfate in Gifford's solution and with the acetate in Feldman's solution. Heavy precipitates indicative of zinc hydroxide formation appeared in solutions of all salts in both buffer systems at *p*H 7.19 and 7.35. After three days nearly every solution showed at least a slight amount of flocculent precipitate which on microscopic examination showed cellular structures which appeared to be molds and yeasts as previously reported by Skauen and Burroughs (15). Traces of basic salts were found in mixtures of *p*H 6 with more noticeable amounts above *p*H 6.4. Zinc sulfate and nitrate gave the slightest amount of basic salt formation on standing.

Zinc sulfate gave precipitates at *p*H 6.8 and above in barbital-sodium barbital buffers containing no sodium citrate, the concentration of zinc sulfate being 0.0075 *M*. In presence of 0.5% sodium citrate, as in modified buffers B and C, the solutions of zinc sulfate remained clear over the *p*H range 6.6 to 9.5.

A search of the literature indicated that the following compounds have been reported as preventing precipitation of zinc salts in neutral or alkaline solution: sodium citrate (11, 12, 16, 17), sodium acetate (10), sodium tartrate (18), ammonium chloride (4, 5, 19), aminoacetic acid (20), and *o*-, *m*-, and *p*-aminobenzoic acids (21).

Alkaline Zinc Collyria.—Tests were made with various substances to obtain clear, alkaline zinc solutions suitable for use as collyria. The quantities of acid and alkaline buffer substances required to give the desired *p*H were obtained by interpolation of neutralization curves of the acid and alkaline buffer solutions, each of which contained the same concentration of zinc salt and stabilizing agent and sufficient neutral salt or other compound to attain approximate isotonicity with the tear secretion. The quantity of substance required to make the solutions isotonic was calculated by the osmotic factor method (22). In each case the freezing point was determined by means of the Bartley apparatus (23). The formulas which follow appear satisfactory for ophthalmic use on the basis of *p*H and isotonicity but clinical tests are needed to determine how the alkaline zinc collyria compare therapeutically with presently used zinc collyria which are slightly acid in reaction. All the formulas are stated in terms of the number of grams of each solute in 100 cc. of solution.

Formula 1

Barbital	0.15
Sodium Barbital	0.25
Sodium Citrate	0.50
Sodium Chloride	1.00
Zinc Sulfate	0.25
Distilled Water, *q. s.*	100.00

Formula 1: *p*H 7.48 at 28°. F. p. = −0.78°. Gave no sensation in eyes. No precipitation after one month. Mold growth abundant.

Formula 2

Barbital	0.26
Sodium Barbital	0.12
Sodium Citrate	3.18
Zinc Sulfate	0.25
Distilled Water, *q. s.*	100.00

Formula 2: *p*H 7.40 at 27°. F. p. = −0.65°. Gave no sensation in eyes. Remained clear even after several months.

Formula 3

Barbital	0.26
Sodium Barbital	0.19
Potassium Sodium Tartrate	4.14
Zinc Sulfate	0.25
Preserved Distilled Water	100 00

Formula 3: *p*H 7.38 at 25°. F. p. = −0.74°. Gave a slight stinging sensation in eyes. Mold growth in seven days, no other precipitation after several months.

The preserved distilled water was made by dissolving 0.026% of methyl *p*-hydroxybenzoate and 0.014% propyl *p*-hydroxybenzoate in distilled water.

Formula 4

Ammonium Chloride	0.60
Sodium Barbital	0.75
Sodium Chloride	0 48
Zinc Sulfate	0.25
Preserved Distilled Water	100.00

Formula 4: *p*H 7.52 at 28°. F. p. = −1.09°. Gave a slight stinging sensation in eyes. Remained clear even after several months.

Formula 5

Hydroxylamine Hydrochloride	0 25
Sodium Barbital	1.48
Potassium Chloride	0.78
Zinc Sulfate	0.25
Preserved Distilled Water	100.00

Formula 5: *p*H 7.49 at 28°. Remained clear for several weeks.

Formula 6

Aminoacetic Acid	0.15
Sodium Barbital	0.41
Sodium Chloride	1.10
Zinc Sulfate	0.25
Preserved Distilled Water, *q. s.*	100.00

Formula 6: *p*H 7.33 at 28°. F. p. = −0.78°. Gave no sensation in eyes. Remained clear even after several months.

Formula 7

Aminoacetic Acid	0.15
Sodium Barbital	0.30
Dextrose	7.73
Zinc Sulfate	0.125
Preserved Distilled Water, *q. s.*	100.00

Formula 7: *p*H 7.59 at 25°. F. p. = −0.85°. Gave no sensation in eyes. Remained clear on standing several months.

Formula 8

Citric Acid	0.54
Sodium Borate	1.70
Zinc Sulfate	0.25
Potassium Chloride	0.86
Preserved Distilled Water, *q. s.*	100.00

Formula 8: *p*H 7.60. F. p. = −0.88°. Gave slight stinging sensation in eyes. Stable after several months.

Formula 9

Aminoacetic Acid	2.12
Sodium Borate	0.63
Boric Acid	0.83
Zinc Sulfate	0.25
Preserved Distilled Water, *q. s.*	100.00

Formula 9: *p*H 7.58 at 25°. F. p. = −0.91°. Gave no sensation in eyes. Stable after several months.

Formula 10

Aminoacetic Acid	0.30
Sodium Borate	1.50
Dextrose	4.16
Zinc Sulfate	0.25
Preserved Distilled Water, *q. s.*	100.00

Formula 10: *p*H 7.44 at 26°. F. p. = −0.77°. Gave no sensation in eyes. Remained clear even after several months.

DISCUSSION

Several of the formulas yield solutions having a *p*H of about 7.6 at room temperature but these *p*H values drop to about 7.4 at body temperature.

In Formulas 4 to 7, inclusive, no barbital is included in the formula since the acidity of aminoacetic acid, ammonium chloride and hydroxylamine hydrochloride, respectively, liberates sufficient barbital to give the desired buffer action. In Formulas 2, 3, and 9 the stabilizer is used in sufficient quantity to act also as an isotonic adjusting agent.

Formulas 8 to 10, inclusive, comprise borate buffers in which the zinc is kept from precipitating in alkaline solution by the presence of citric acid or aminoacetic acid. Formula 10 illustrates the ability of dextrose to increase the ionization of boric acid so that it becomes a stronger acid, as the addition of dextrose reduced the *p*H from 8.3 to 7.4.

SUMMARY

1. A barbital-sodium barbital buffer was developed as a possible ophthalmic buffer having maximum buffer capacity at the approximate *p*H of the tear secretion.

2. Formulas have been developed for solutions of zinc salts in which the use of stabilizers, such as citrates, tartrates and aminoacetic acid, prevents the precipitation of zinc at *p*H 7.4, which is the approximate *p*H of the tear secretion.

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The Analysis of Ipecac—Drug, Tincture, and Fluidextract*

By FLOYD A. WHIPPLE and J. M. WOODSIDE

Several modifications of the official methods of assay for ipecac and ipecac preparations have been devised and tested. These modifications are described and the results obtained by each are recorded. A general method of assay, applicable to ipecac and ipecac preparations and with fewer inherent sources of error than those of any of the official methods, is recommended.

IPECAC and its preparations have had official use in medicine for many years. The present methods of assay have remained practically the same for the last several revisions of the U. S. P. and N. F.

However, different operators (1), at times, have expressed various difficulties in the extraction of the alkaloids by the official methods, especially with the N. F. tincture. The U. S. P. XIII method (2) for the drug and fluidextract involves, as do certain other official assays, the transfer of an aliquot part of a very volatile solvent, ether. In this method, 10 Gm. of drug or 10 cc. of the F.E. absorbed on filter paper or asbestos is macerated overnight in 100 cc. of ammonia-ether solution. After considerable shaking and allowing to stand overnight, a 50-cc. aliquot of the ammonia-ether solution (very difficult to measure accurately!) is then taken, which theoretically represents 5 Gm. of drug or 5 cc. of the F.E. Even when this operation is carried out as rapidly as possible, and at controlled temperatures, it seems that some of the ether cannot help but be vaporized in the shaking process, and therefore the ether solution obtained will be somewhat concentrated. The method of the A.O.A.C. (3) avoids this by making an acid dilution and taking the aliquot part of the acid solution and not of an ether solution of the alkaloids. Digangi and Trupp (1) also suggested preparation of an acid filtrate for apparently the same reason. They expressed the fact that the difficulty with the N. F. tincture (4) method is mostly from a manipulative standpoint. We, too, usually find it practically impossible to obtain a satisfactory result in this case without enduring much emulsification and using ether in a volume too great for practical purposes. This prompted the running of several "research" experiments—using the official assay methods and then applying some of the suggested modifications to see if a method of assay could be obtained which is more readily workable and whereby check results can be obtained.

* Received Sept. 5, 1947, from the Chemical Control Laboratories, Eli Lilly and Company, Indianapolis, Ind.

EXPERIMENTAL

I. Ipecac (Drug)

(a) **Soxhlet Extraction.**—Place 5 Gm. of the drug in an extraction thimble, insert the thimble in a Soxhlet extractor, as directed in the U. S. P. XIII, page 677, paragraph "C" and, after macerating with ammonia T. S. overnight, extract with ether for four hours, then continue as in the U. S. P. XIII method, with the acid extractions.

(b) **Percolation.**—Place 5 Gm. of the drug in a small percolator. Macerate overnight with ammonia T. S., then percolate the next day with ether until the drug is exhausted, and continue as in the U. S. P. XIII method, extracting the ether with acid, etc.

(c) **Maceration.**—Macerate 5 Gm. of the drug in a glass-stoppered flask overnight using ammonia T. S. and 50 cc. of ether. Shake the mixture for one hour, then decant the ether through a filter. Wash the residual drug with several portions of ether, adding these through the filter. Combine the total ether extracts, evaporate to about 50 cc., and continue the assay as in the U. S. P. XIII, extracting the ether with acid, etc.

(d) **U. S. P. XIII Method.**—(As is.)

SERIES NO. I.—RESULTS OF IPECAC ASSAYS BY METHODS DESCRIBED ABOVE

Drug No. 1			
	Method	Result, %	Check Result, %
(a)	Soxhlet	2.65	2.60
(b)	Percolation	2.40	...
(c)	Maceration	2.58	2.66
(d)	U. S. P. XIII	2.75	2.88

Comments.—(a) The Soxhlet method works very well with no manipulative difficulty encountered.

(*b*) The Percolation method needs a large volume of ether and requires a longer time period than the others; also more cumbersome.

(*c*) The Maceration method is not too practical. The results check with the Soxhlet method, although the method of extraction is not as easy as in the Soxhlet method.

SERIES NO. II.—RESULTS OF IPECAC ASSAYS

Method	Operator No. 1, %	Operator No. 2., %
Drug No. 2		
(*a*) Soxhlet	2.95 2.89	2.88
(*b*) U. S. P. XIII	3.52 3.18	3.09
Drug No. 3		
(*a*) Soxhlet	2.79 2.85	2.86 2.89
(*b*) U. S. P. XIII	2.97	3.03

Comments.—The results show that the Soxhlet method is superior to the U. S. P. XIII method in that it is easier to manipulate, the results seem more readily reproducible, and it is subject to no apparent source of error.

Proposed Assay Method for Ipecac Drug

Accurately weigh 5 Gm. of the ground sample, place it in an extraction thimble and insert the thimble in a suitable Soxhlet extractor. Add enough ether to thoroughly cover the drug and allow to stand five minutes, stirring occasionally. Add 3 cc. of stronger ammonia T. S. and mix thoroughly. Allow to macerate overnight, then cover the drug with a pledget of cotton, pack thoroughly, and extract with ether for four hours, or until completely extracted as shown by a suitable test.

Transfer the ether extracts to a separatory funnel, rinse the container with a small amount of ether, and continue as in the U. S. P. XIII assay method, page 278, second paragraph, beginning with "Completely extract the alkaloids," etc.

II. Fluidextract of Ipecac, U. S. P. XIII

(a) **N. F. VIII Method for Tincture Applied to F.E. (Triple Shake-Out).**—Here the N. F. VIII Tincture Ipecac method was followed using a 5-cc. sample and not evaporating, but adding 5 cc. of water.

(b) **Acid Filtrate Method (1).**—Dilute 10 cc. of F.E. with 10 cc. of dilute sulfuric acid and add enough distilled water to make exactly 100 cc. After filtering, transfer exactly 50 cc. of this dilution to a separatory funnel and extract twice with ether. Combine these ether extracts and wash with 10 cc. of distilled water. Add the water to the acid solution and discard the ether. Add enough ammonia to make the solution alkaline, then extract with 50-cc. portions of ether, evaporate these, and titrate the alkaloids in the usual manner.

(c) **Modification of the N. F. VIII Method.**—Dilute 5 cc. of F.E. with 50 cc. of distilled water in an automatic extraction apparatus (Palkin-Watkins), add 5 cc. of ammonia T. S., and extract with ether for four hours. Carefully transfer the ether to a separatory funnel, extract with acid, and continue as in the U. S. P. method.

(d) **U. S. P. XIII Method.**—Run as in U. S. P. XIII.

SERIES NO. III.—RESULTS OF FLUIDEXTRACT ASSAYS

Method	Result (Gm. Alkaloids per 100 Cc.)	Check Result (Gm. Alkaloids per 100 Cc.)
F.E. No. 1		
(*a*) Triple shake-out (N. F. VIII)	2.11	1.84
(*b*) Acid filtrate	2.40	2.26
(*c*) Modified N. F. VIII	2.13	2.17
(*d*) U. S. P. XIII	1.91	2.20

Comments.—(*a*) The triple shake-out method is long and tedious. Several emulsions were experienced.

(*b*) The acid filtrate method is more readily workable but not in line with any comparable method for the drug. The results are higher than by other methods, probably because there is no preliminary series of ether shake-outs.

SERIES NO. IV.—RESULTS OF FLUIDEXTRACT ASSAYS

Method	Result (Gm. Alkaloids in 100 Cc.) Operator No. 1	Check Result (Gm. Alkaloids in 100 Cc.) Operator No. 2
F.E. No. 2		
Modified N. F. VIII	2.086	2.11
U. S. P. XIII	2.16	2.04

Comments.—We believe the modification of the N. F. VIII Tincture method, using automatic extraction for the first series of alkaline-ether shake-outs, is a method superior to the method of the U. S. P. XIII for the F.E. because:

(*a*) It eliminates taking an aliquot part of a volatile solvent, ether.

(*b*) The material does not have to stand overnight and an assay can be run in about five hours (over-all time), actually using less than an hour of the analyst's time.

(*c*) It gives more concordant results.

(*d*) It eliminates absorption of the F.E. on filter paper.

(*e*) It is very easy to manipulate.

Suggested Revision Method for the Assay of Fluid Extract Ipecac, U. S. P.

Place exactly 5 cc. of Fluidextract Ipecac in a Palkin–Watkins automatic extractor and add 50 cc. of distilled water. Make the solution in the extractor distinctly alkaline with stronger ammonia water and carefully add the proper amount of peroxide-free ether. Connect the extractor to a condenser and extract for four hours. Occasionally disconnect the extractor from the condenser and agitate the lower layer by raising and lowering the center extraction tube. After the four-hour extraction period, disconnect the extractor and carefully transfer the ether to a 250-cc. separator. Wash the extraction flask with two small portions of ether and add these to the separator. Extract the alkaloids from the ether solution by shaking with four 25-cc. portions of 2% sulfuric acid. Now make the acid extractions alkaline by adding a slight excess of stronger ammonia and extract with five 40-cc. portions of peroxide-free ether. Filter the ether into a 250-cc. beaker. Evaporate until the volume is about 2 cc., add 5 cc. alcohol and 20 cc. 0.05 N H_2SO_4. Warm on the steam bath for a few minutes to expel the last traces of ether, then cool and titrate with 0.02 N NaOH, using Methyl Red as the indicator.

Each cc. 0.02 N H_2SO_4 = 4.80 mg. Ipecac alkaloids.

III. Tincture of Ipecac N. F. VIII

(*a*) The acid filtrate method [as described by Digangi and Trupp (1)].

(*b*) The U. S. P. XIII F.E. method modified in that 50 cc. of the Tincture is used as a sample.

(*c*) The N. F. VIII method modified as in the assay of the F.E., using automatic extraction for the first series of alkaline-ether shake-outs.

(*d*) N. F. VIII as prescribed.

SERIES NO. V.—RESULTS OF TINCTURE ASSAYS

Method	Result (Gm. Alkaloids per 100 Cc.)	Check Result (Gm. Alkaloids per 100 Cc.)
(*a*) Acid filtrate	0.245	0.232
(*b*) Modified U. S. P. XIII	0.228	0.145
(*c*) Modified N. F. VIII	0.211	0.205
(*d*) N. F. VIII	0.107	0.185
		0.201

Comments.—(*a*) By the acid filtrate method the results were unusually high.

(*b*) The modified U. S. P. XIII method had too much liquid to be properly absorbed; seemingly not practical for Tincture.

(*c*) The modified N. F. VIII method was easiest to work—no emulsification and good duplication of results.

SERIES NO. VI.—RESULTS OF TINCTURE ASSAYS
Tincture No. 2

Modified N. F. VIII Method

	Gm. Alkaloids per 100 Cc. Operator No. 1	Gm. Alkaloids per 100 Cc. Operator No. 2	N. F. VIII Method as Is
Sample No. 1	0.226	0.222	0.209
Sample No. 2	0.210	0.215	0.197

Comments.—Here, as in the case of the F.E., the modification of the N. F. VIII method, using automatic extraction for the first series of alkaline-ether shake-outs, is superior to the present N. F. VIII method since it creates less manipulative difficulty.

Suggested Revision Method for the Assay of Tincture Ipecac, N. F.

Place exactly 50 cc. of Tincture Ipecac in a 250-cc. beaker and evaporate on a steam bath until the volume is reduced to 20 cc. Carefully transfer the 20 cc. to a Palkin–Watkins continuous extractor and rinse the beaker with two 20-cc. portions of distilled water, placing these portions in the extractor. Make the solution in the extractor distinctly alkaline with stronger ammonia water; carefully add the proper amount of peroxide-free ether. Connect the extractor to a condenser and extract for four hours. Occasionally disconnect the extractor from the condenser and agitate the lower layer by raising and lowering the center extraction tube. After the four-hour extraction period, disconnect the extractor and carefully transfer the ether to a 250-cc. separator. Wash the extraction flask with two small portions of ether and add these to the separator. Extract the alkaloids from the ether solution by shaking with four 25-cc. portions of 2% sulfuric acid. Now make the acid extractions alkaline by adding a slight excess of stronger ammonia and shake out with four 50-cc. portions of peroxide-free ether. Filter the ether through a filter paper into a 250-cc. beaker. Evaporate until the volume is about 2 cc., add 5 cc. alcohol, 20 cc. 0.05 N H_2SO_4, heat on the steam bath for a few minutes to expel the last traces of ether, then cool and titrate with 0.02 N NaOH, using Methyl Red as the indicator.

Each cc. 0.02 N H_2SO_4 = 4.80 mg. Ipecac alkaloids.

DISCUSSION

The over-all time factor in this revised method might possibly be a little longer in the Tincture assay than the present official method, but the actual man-time consumed will usually be less. Results obtained by using this method are slightly higher than those found in the official method for Tincture Ipecac.

The method suggested will give clean-cut separations with the aqueous and immiscible solvent phase, thereby eliminating the trouble usually encountered with emulsions.

In each case we have substituted automatic extraction for the first series of ether shake-outs. We believe this is advantageous since it is here that the alkaloids are separated from the other drug constituents and it is here, therefore, that complete extraction is essential, since manipulations by the official method are most difficult at this stage. We also believe that it is necessary to start out with a series of *alkaline ether extractions* in each case and not an acid dilution or acid filtrate. In this way, further purification of the alkaloids is obtained since the results in the acid filtrate method experiments are consistently higher, probably due to impurities present which a preliminary ether extraction would eliminate. We have successfully used the Soxhlet extraction method as suggested for the drug in analysis of Tablets of Ipecac and of an Ipecac Alkaloids Compound, which is in reality a concentrated mixture of the alkaloids absorbed on Lloyd's Reagent.

SUMMARY AND CONCLUSION

Various difficulties have been encountered in the analysis of Ipecac and its Fluidextract and Tincture by the official methods.

It is the desire of the authors of this pape to develop one general method that will ap ply not only to the Ipecac drug, but also t those pharmaceutical preparations which ar made from Ipecac.

We have tried several modifications, i each case eliminating the methods which w found least desirable and finally emergin with methods which, by not too great modification of the official methods, yiel results which are more easily obtained an more readily reproducible.

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Influence of Preliminary Administration of Insulin or of Epinephrine Hydrochloride Upon the Fatal Dose of Sodium Evipal in Albino Mice* †

By HARALD G. O. HOLCK‡

Under conditions of this experiment using 104 mice, results show that neither epinephrine hydrochloride nor insulin, preliminarily administered, alter significantly the fatal dose of Sodium Evipal.

IN 1945 Reinhard (1) reported that insulin given to white mice twenty minutes prior to Sodium Evipal prolonged the hypnosis induced by 100 mg. per Kg. of the latter drug by 67 to 89 per cent and that similarly administered epinephrine hydrochloride increased the sleeping time by 78 per cent.

In view of the increased use of intravenous anesthesia with ultrashort acting barbiturates and because of the common use of both insulin and of epinephrine, an experiment was undertaken to ascertain what effect, if any, doses similar to those employed by Reinhard would have upon the average fatal dose (LD_{50}) of Sodium Evipal in albino mice.

EXPERIMENTAL

The mice used were nonfasted male albino Swiss mice, weighing 18 to 28 Gm. The insulin was diluted with 0.9% sodium chloride solution so that 1 cc. contained one unit. One unit per Kg. of bodyweight was then administered into a lateral thigh muscle and twenty minutes later followed by Sodium Evipal given under the skin of the lower back; 0.91 unit per Kg. had previously been found to produce convulsions in 50% of similar mice under insulin assay conditions. The Sodium Evipal solu-

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Presented to the Scientific Section of the A. PH. A., Milwaukee meeting, August, 1947.
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‡ We are indebted to the Winthrop Chemical Company, Inc., for supplying the Sodium Evipal, and to Mr. David Russell for aid in the tests.

TABLE I.—COMPARISON OF SODIUM EVIPAL MORTALITIES IN MALE ALBINO MICE FOLLOWING MEDICATION WITH EPINEPHRINE HYDROCHLORIDE AND WITH INSULIN, RESPECTIVELY[a]

Sodium Evipal, Mg./Kg.	Mortalities		
		Pretreated with	
	Controls	Epinephrine	Insulin
375	1/13	0/13	1/13
447	4/13	1/13	1/13
533	3/13	1/13	2/13
635	3/13	5/13	5/13
757	6/13	8/13	11/13
903	8/13	10/13	13/13
986	10/13	9/13	13/13
1077	13/13	13/13	12/13
Total:	48/104	47/104	58/104
LD_{50} and S.E.,[b] mg./Kg.	730 ± 56	735 ± 45	640 ± 67

[a] Nonfasted male Swiss albino mice, injected subcutaneously with 5% solution of Sodium Evipal. One unit of insulin, or 0.04 mg. of epinephrine hydrochloride, or an equivalent amount of 0.9% sodium chloride solution per Kg. was injected intramuscularly 20 minutes prior to the barbiturate administration. The room temperature was 27 ± 1°.

[b] These estimates of the LD_{50} and their approximate standard errors were made by Dr. Lloyd C. Miller using a graphical procedure (3).

tion was 5% in distilled water and doses covered practically the whole fatal range. In case of epinephrine hydrochloride, 0.04 mg. per Kg. of mouse, using 2 cc. of a 50,000 dilution in 0.9% solution of sodium chloride was administered intramuscularly twenty minutes before Sodium Evipal. Although a considerable part of the epinephrine action may be over in twenty minutes, we chose the procedure of Reinhard (1), because his results indicated that with the large dose used—equivalent to 3 mg. in an adult person—the mice were clearly affected by epinephrine after twenty minutes as indicated by the prolonged hypnosis following Evipal administration. In case of the control mice, 1 cc. of 0.9% solution of sodium chloride per Kg. was similarly administered intramuscularly twenty minutes ahead of the Sodium Evipal. After medication the mice were kept individually in large glass beakers and the room temperature kept as close as possible to 27°. This is of particular importance because Chance (2) recently has reported that the toxicity of certain sympathomimetic amines, including epinephrine, was much greater in mice kept in groups after injection than in those kept in individual containers at our experimental temperature. The doses of Sodium Evipal, which started with 375 mg. per Kg., were increased in steps of 19.2%, except that in case of the dose of 986 mg. per Kg., the difference is only half the logarithmic increase, or 9.2%; this extra dose was employed to increase the number of animals in the range close to the surely fatal dose. A total of 104 mice were injected in each series and the results are presented in Table I.

Although our main concern was to study the influence of insulin and of epinephrine upon Evipal mortality, records were also kept of the recovery times until the mortality recording was discontinued at 10 p.m., after which the remaining mice were left for further observation on the following morning. Judging from the recovery times of about the first three-fourths of the mice only, we were unable to find any constant prolongation of depression due to insulin or to epinephrine in the fatal dosage range.

SUMMARY

Our results show that preliminary medication with a substantial dose of epinephrine hydrochloride did not influence the average fatal dose of Sodium Evipal when the latter was given twenty minutes later. Although the LD_{50} of Sodium Evipal is somewhat lower following medication with insulin, it is only 12.2 per cent below the control value; also, because the mortalities with the lower 4 or 5 doses are quite similar in the two cases and because the only mouse to survive 1077 mg./Kg. had been pretreated with insulin, it would appear that insulin does not significantly alter the fatal dose of Sodium Evipal under the conditions of our experiment.

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Note

Note on a Color Reaction for Shark-Liver Oil*

By LAWRENCE S. MALOWAN

For the purpose of detecting vitamin A in fish-liver oil by chemical means the reaction of Carr and Price (1) is the most generally used and is based upon the formation of a blue color when antimony trichloride is added to a solution of the vitamin-bearing oil. Recently proposed were glycerol 1,3-dichlorhydrin (2) and activated earth (3) as substitutes for antimony trichloride as colorimetric reagent.

EXPERIMENTAL

It was found that titanium tetrachloride in organic solvents produces a deep and stable color when added to a solution of shark-liver oil. Antimony trichloride produces no change when added to a solution of a fatty oil free from vitamin A or when added to solutions of fish-liver oil, the known vitamin A reaction. On the contrary, titanium tetrachloride in organic solvents mixed with fatty oils causes the formation of slightly red or brown colors. We investigated peanut, cottonseed, sesame, olive, castor, and linseed oils, all of which behave in the same way. Specific, however, is the reaction with shark-liver oil through the color effect shown.

Conduct of the Test.—If 1 ml. of a solution of 10% titanium tetrachloride in acetylene tetrachloride is mixed with 5 ml. of a 10% solution of shark-liver oil in chloroform, it causes a brilliant color display by producing at first a blue color which soon changes to a deep red violet. Even in presence of an excess of another fatty oil the reaction of shark-liver oil is distinctly visible, and the color does not fade for at least three hours.

DISCUSSION

Titanium tetrachloride in a pure state is not a very convenient reagent because of the dense fumes it produces, but it is easy to handle when dissolved in organic solvents, from which was chosen acetylene tetrachloride because of its high boiling point and the fact that, when mixed with the mentioned chloride, it does not produce any more fumes than hydrochloric acid. The reagent is quite stable; however, it contains some yellow precipitate.

The mechanism of the reaction described was studied more closely by performing tests with the saponifiable and unsaponifiable fractions of shark-liver oil. The result has been that with neither fraction could be obtained any result identical to the effect produced by the shark-liver oil itself. The saponifiable fraction gives no specific reaction while the unsaponifiable portion produces a color similar to that caused by antimony trichloride. Because of the formation of considerable precipitate the titanium reagent is unsuitable to be used for quantitative purposes. Carotene gives a blue color while cholesterol and phytosterol solutions do not show any change. Vitamin D in the form of viosterol or calciferol, however, gives a deep brown-red color with the titanium tetrachloride reagent, while antimony trichloride leaves the solution of vitamin D nearly colorless. By this factor the presence of vitamin D may be verified in medical preparations. Twenty-five tablets of Dical-D were extracted with chloroform for forty-eight hours. The extract was evaporated and the yellow residue redissolved in chloroform and mixed with the titanium tetrachloride solution. It developed a permanent deep red color. A similar experiment was carried out with Sterogyl; however, the vitamin solution in oil was primarily saponified. The unsaponifiable residue gave with the reagent a color similar to that obtained with the extract of Dical-D. The color developed by shark-liver oil with the titanium tetrachloride reagent is probably not due to its vitamin D content because it was negligible in the oils employed in these experiments.

SUMMARY

A simple color test for the detection of shark-liver oil, employing titanium tetrachloride, is described. This reagent is specific and allows to detect 0.5 ml. of the oil in presence of an excess of other fatty oils.

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(1) Carr, F. H., and Price, E. A., *Biochem. J.*, 20, 497 (1926).
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JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXVII | *MARCH, 1948* | NUMBER 3 CONSECUTIVE NO. 6

An Investigation of *Piscidia Erythrina* (Jamaica Dogwood)*

By CHRISTOPHER H. COSTELLO and CALVIN L. BUTLER†

The chemical, pharmacological, and clinical history of Jamaica dogwood is reviewed. Preparations of all parts of Piscidia were found to exert appreciable uterine depressant action both *in vitro* and *in vivo* on laboratory animals. The root bark extracts were the most potent, and petroleum ether was found to be the most effective solvent for the depressant principle. The drug is apparently nontoxic to rats fed massive oral doses.

WHILE investigating the actions of various drugs employed in uterine sedative formulas (1, 2), it was found that *Piscidia erythrina* Linn., commonly known as Jamaica Dogwood, was a potent depressant in our hands on the uteri *in vitro* and *in vivo* of various laboratory animals and was worthy of further investigation. The present report concerns the study of the uterine activity of this plant, and the chemical work done for the purpose of tracing the potent substances, with a view to isolation. The latter was not intended to be complete.

HISTORY

Jamaica Dogwood is a leguminous tree, native to the West Indies and the adjacent mainland, and also found in Florida, Texas, Mexico, and the northern portion of South America (3). The plant has a long history of use by the natives of Jamaica as a hypnotic and fish poison. The taste or edibility of the fish are in no way affected by this process (3, 4). It is also an ingredient of the Mexican native sedative tea called "Sinicuichi" together with two other plants (5).

Although the drug has never been official in any American compendium, it has appeared in several European pharmacopœias. Piscidia bark and its fluidextract were admitted in 1930 to the Supplement of the German Drug Book, Vol. 5, 1930 (4).

The chemical investigation originates with Hart (6) who, in 1883, obtained a neutral crystalline substance on treating the fluidextract with slaked lime. He believed this to be the active principle and named it piscidin. This was credited by Berberich (7) as having the formula $C_{29}H_{24}O_8$, melting at 192°.

Freer and Clover (8) have shown that the substance known as piscidin is a mixture of two glycosides. The first of these had a composition of $C_{23}H_{20}O_7$, forming colorless, highly refracting rectangular crystals, melting at 201°. The second had a composition of $C_{22}H_{18}O_6$, forming fine yellow

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†We wish to acknowledge the technical assistance of Lucille E. Mullen and Ursula E. Gassiraro.

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Although the drug has never been official in any American compendium, it has appeared in several European pharmacopœias. Piscidia bark and its fluidextract were admitted in 1930 to the Supplement of the German Drug Book, Vol. 5, 1930 (4).

The chemical investigation originates with Hart (6) who, in 1883, obtained a neutral crystalline substance on treating the fluidextract with slaked lime. He believed this to be the active principle and named it piscidin. This was credited by Berberich (7) as having the formula $C_{29}H_{24}O_8$, melting at 192°.

Freer and Clover (8) have shown that the substance known as piscidin is a mixture of two glycosides. The first of these had a composition of $C_{23}H_{20}O_7$, forming colorless, highly refracting rectangular crystals, melting at 201°. The second had a composition of $C_{22}H_{18}O_6$, forming fine yellow

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needles, melting at 216°. Piscidic acid, having a composition of $H_2 \cdot C_{11}H_{10}O_7$ was reported in the aqueous extract, forming acicular crystals melting at 182–185°.

An exhaustive chemical investigation of the bark was conducted by Danckwortt and Schütte (9) in 1934. They obtained from the petroleum ether extract a crystalline alcohol of the paraffin group ($C_{17}H_{36}O$, m. p. 79°) and a crystalline monohydric alcohol belonging to the sterols ($C_{27}H_{48}O_2$, m. p. 145°). From the ether extract was obtained two crystalline substances, $C_{15}H_{14}O(OH)_2(OCH)_3CO_2H$, m. p. 158°, and an amorphous substance of undetermined composition, m. p. 70°. A characteristic constituent was found to be a water-soluble glycoside of saponin character, yielding glucose and galactose. Both acidified alcohol, and trichloroethylene extracted an alkaloidal substance, m. p. 86–87°, as a yellowish white amorphous powder. The latter employed the stem bark, whereas the previous authors used the root bark.

The earliest work on the pharmacodynamics of Jamaica Dogwood appears to be that of Ott (10, 11) and Nagle (12) who both found that it had narcotic properties, caused dilation of the pupils, increased secretion of sweat, and caused rise of blood pressure in lower animals.

Pharmacological research by Pellocani (13) and Cushny (14) showed that the mixture designated as piscidin could not be the active principle.

Pilcher and his associates (15), in 1916, found that Piscidia diminished the contractions of the excised intestinal strip of the rabbit and the excised uterus of the guinea pig but did not affect arterial smooth muscle. The action of the drug on lessening the amplitude and rate of contractions of the isolated guinea pig uterus was confirmed by Macht (16).

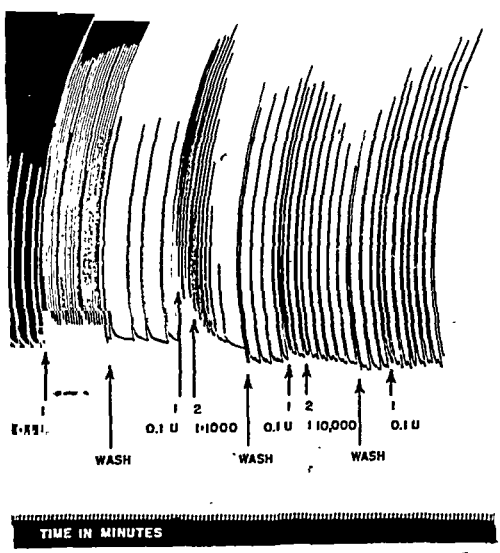


Fig. 1.—Effect on rat (anestrus), isolated uterus, left horn, *in vitro*. Drugs: 1, Pitocin; 2, Piscidia fluidextract, dealcoholized, buffered, not filtered.

Pittenger and E'We (17) reported that the fluidextract administered orally to dogs had an action similar to cannabis but required approximately seventeen times as much to produce the same degree of incoordination. Piscidin obtained from the fluidextract represented 55% to 62.5% of its activity. This is in contradiction to the earlier reports of Pellocani and Cushny.

Pharmacological investigation of the saponin isolated by Schütte from the tree bark was conducted by Kofler (18). The lethal dose by intravenous injection was 0.075 mg. per Gm. of mouse. Oral doses up to 1.5 mg. per Gm. of mouse, however, had no effect.

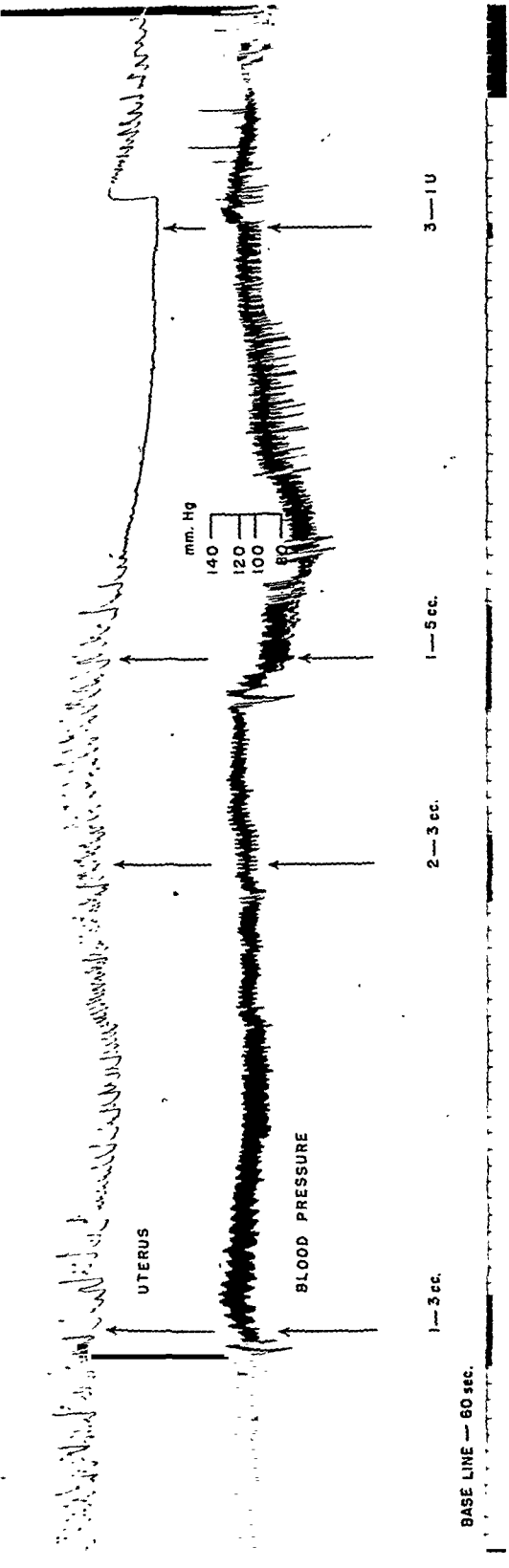
An alcohol-soluble principle isolated from the petroleum ether extract was found by Hauschild (4) to be toxic to fish in dilutions of 1:80,000,000. The substance was strongly poisonous to rats and rabbits when administered parenterally, causing death by respiratory paralysis, but was nontoxic when administered orally in doses up to 5 mg. per Kg.

The effect of this drug on man as a remedy has not as yet been fully determined. Various investigators have reported good results from its use as an anodyne in neuralgia, nervous insomnia, cough, and dysmenorrhea. Ott (10, 11) first recommended Jamaica Dogwood in the treatment of chronic cough. Firth (19), in 1883, reported excellent results with the drug in delirium tremens. Payne (20) made a similar report. In the same year, Seifert (21) tried it clinically at the Julius Hospital in Vienna, and found it to be valuable in phthisis, causing a diminution of night sweating and also of cough, without any side effects. Later, Hamilton (22) called the attention of the profession to Jamaica Dogwood as a powerful sedative and analgesic, without the side effects that follow the use of opium and its derivatives. Peyer (23) used the bark as a sedative in the form of a tea. The most recent clinical contribution is that of LeClerc (24) who reported its value in the relief of neuralgia and whooping cough.

Earlier investigators report the drug as consisting of the root bark. Upon examination of the commercial product and correspondence with well-informed sources in Jamaica, it proved to be the stem bark, a by-product of lumbering operations. The commercial product used in this investigation was supplied by S. B. Penick and Company in 1947. Material collected locally from all parts of the plant was supplied by Mr. Arthur R. Marshall of Miami, Florida, and Mr. Cecil B. Facey, Kingston, Jamaica. All material used was authenticated pharmacognostically by Dr. Heber W. Youngken, Boston.

EXPERIMENTAL

Pharmacological Methods.—Experiments upon isolated tissues were carried on according to the Magnus method as described by Burn (25). The isolated uterus of the rat was used by preference



since results were more easily reproducible and the various stages of the estrus cycle could be easily determined. The majority of the *in vivo* studies were based upon modification of the Barbour method. Tracings were made simultaneously of uterine contractions, blood pressure, and respiration. Drugs were given intravenously. A number of experiments employing the intrauterine balloon method were also made upon the *Rhesus macacus* monkey. This method is a modification of those employed by Bickers (26) and Woodbury, *et al.* (27).

Solutions used *in vitro* and *in vivo* were usually dealcoholized, buffered, and filtered. However, in certain cases *in vitro*, this procedure was omitted, since we found by experiment that the very small volumes of the various solvents for the drug showed no effect upon the tissue. Detailed procedures are described in previous papers (2, 28).

Fluidextract.—The fluidextract was prepared from commercial bark by the U. S. P. method type process A using 38.5% alcohol as a menstruum, and was first dealcoholized and buffered to *p*H 7.5 before use in any experiment. In a series of twenty *in vitro* experiments with the fluidextract of Piscidia, effective sedation was shown using dilutions ranging from 1:17,500 to 1:20,000. This was likewise found effective in reducing the spasmolytic effect of Pitocin, dilutions of from 1:1000 to 1:10,000 usually materially decreasing the stimulant effect of 0.1 unit (Fig. 1).

In the *in vivo* tests, uterine contractions of the cat were usually reduced using 3 to 5 cc. of the fluidextract. Blood pressure showed irregular drops and rises, especially during injection, while respiration was variable (Fig. 2).

Using the intrauterine balloon method on the monkey, 3 cc. of the fluidextract was found to be inhibiting to uterine motility (Fig. 3).

Alcoholic Extract.—For the purpose of a more complete examination, 10 Kg. of ground commercial bark was exhausted with alcohol in a Soxhlet apparatus. The resulting liquid was freed from alcohol by vacuum distillation to give 3.17% of a dark, granular material. In terms of the powdered drug extracted, the alcoholic extract was found to be effective *in vitro* in a concentration of 1:50,000. Computed in terms of the yield of extract, the concentration is 1:1,600,000.

The dried alcoholic extract was distilled with steam for over six hours, cooled, and filtered.

Volatile Oil.—The distillate yielded a volatile oil collected by means of ether, which amounted to 0.011% of the dried bark. The oil was amber in color with a pungent taste and an acrid odor. Insufficient material was available for further chemical study. Although weakly active, this fraction does

Fig. 2.—Effect on cat, 2.04 Kg. (in estrus), *in vivo*. Drugs (intravenous): 1, fluidextract, buffered, filtered; 2, Alcohol 38.5%; 3, Pitocin.

not constitute the active principle of the bark. In a series of three *in vitro* experiments, the oil showed sedation at a concentration of 1:500 of the drug or 1:100,000 of the oil itself.

Resin.—The residue from distillation of the alcoholic extract contained a physiologically active brown resin amounting to 1.96% of the bark. The resin obtained from commercial bark showed inhibition of uterine activity *in vitro* in a concentration of 1:10,000 in terms of the drug. The resin from the root bark was considerably more potent, producing sedation at a concentration of 1:75,000.

Aqueous Solution.—The filtrate from the resin was completely precipitated with lead acetate solution. The precipitate was suspended in water and decomposed with disodium phosphate; the mixture was filtered and evaporated to dryness. The brown semisolid amounted to 0.073% of the bark and reduced Fehling's solution only after hydrolysis, but was found to be pharmacologically inert.

The filtrate from the lead acetate precipitation yielded an inactive residue amounting to 1.91% of the bark. This was redissolved, and the solution precipitated completely with lead subacetate. This precipitate was also suspended in water and decomposed with disodium phosphate; the mixture was filtered and evaporated to dryness. The brown semisolid obtained amounted to 0.12% of the bark and reduced Fehling's solution only after hydrolysis.

Since only the resin had significant activity on the uterus, and this activity was approximately one-fifth that of the alcoholic extract from which it was derived, one is justified in concluding that the greater portion of the active principle was lost during the process of separation employed.

Successive Extraction.—In order to more closely trace the uterine acting principles with a minimum of loss by chemical methods, 1000 Gm. of ground commercial bark was exhausted in a Soxhlet apparatus employing a series of different solvents in succession.

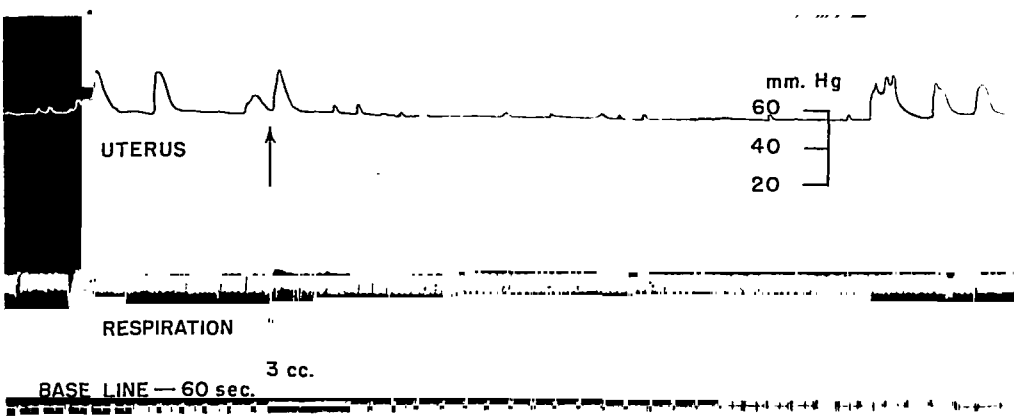


Fig. 3.—Effect on monkey, 4.88 Kg. (in estrus), intrauterine balloon method. Drug (intravenous): Piscidia fluidextract, dealcoholized, buffered, filtered.

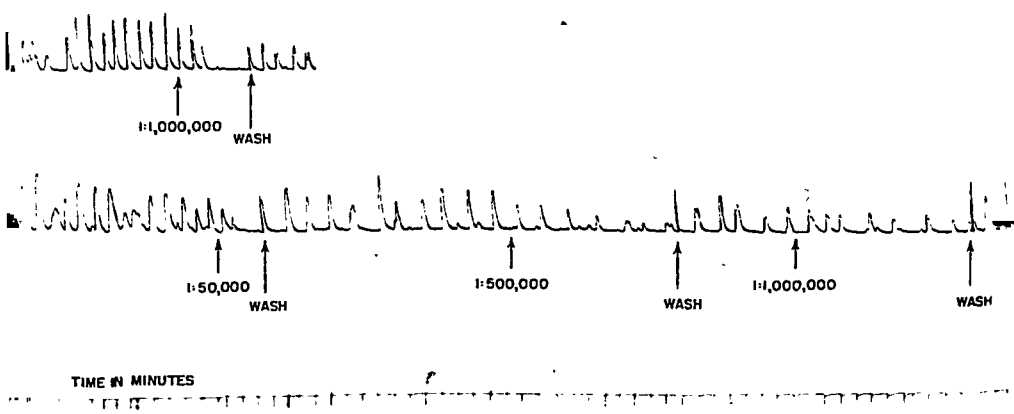


Fig. 4.—Effect on rat (anestrus), isolated uterus, right horn, *in vitro*. Drug: Piscidia, alcohol soluble portion of petroleum ether extract.

Petroleum Ether Extract.—A yellowish green resinous material which separated out from the petroleum ether solution on cooling was found to be significantly active on the uterus. A sufficient quantity was not available for an adequate chemical study. The material amounts to 0.0058% of the bark, is completely soluble in 95% alcohol and alkali, almost insoluble in water, and in its crude state melts between 78–81°. Danckwortt and Schütte (9) report a crystalline alcohol of the paraffin group ($C_{17}H_{36}O$, m. p. 79°) which they also obtained from the petroleum ether extract of this drug. In two *in vitro* experiments, significant inhibition of uterine activity was seen with a concentration of 1:10,000 expressed in terms of the dried drug.

Following the removal of the precipitate, a portion of the petroleum ether solution was distilled to a small volume and then evaporated spontaneously. During the course of the evaporation, a yellowish amorphous substance came down, similar to the precipitate obtained previously. This was not separated, however. The alcohol-soluble portion of the petroleum ether extract represented 0.3733% of the bark. Upon bioassay it was found to have a high order of activity on the uterus. In a series of four *in vitro* experiments, the threshold dose for inhibition of uterine activity was found to be 1:1,000,000 in terms of the dried drug. Larger concentrations showed proportionally more sedation (Fig. 4).

In vivo, filtered and buffered intravenous doses prepared by shaking 11 mg. of this fraction (representing 3 Gm. of drug) with saline solution usually produced marked drops in tonus with some decrease in rate and amplitude (Fig. 5).

Another portion of the petroleum ether extract, upon shaking repeatedly with 1% HCl, yielded no alkaloid to the acid that would give positive tests with the general reagents. Here again, the petroleum ether extract was evaporated and the residue picked up with 95% alcohol. During the course of the evaporation, more of the yellow amorphous material mentioned above was deposited but was not separated. The alcohol solution was again evaporated and picked up in a minimum of alcohol. Upon evaporation of the latter, there was obtained a dark brown viscous material, amounting to 0.123% of the bark. As might be expected, this fraction was of the same order of potency as the precipitate and the alcohol-soluble portion of the petroleum ether extract mentioned above.

In the opinion of the writers, the variance of potency in these three fractions of the petroleum ether extract may be due to the concentration of the yellow amorphous material, and not only to the variance in response of one uterus from another. Much more study is indicated upon the petroleum ether-soluble portion of this drug. It is shown later

Fig. 5.—Effect on cat, 1.65 Kg., *in vivo*. Drug (intravenous): Piscidia, alcohol soluble portion of petroleum ether extract, in saline.

in this paper that the fractions mentioned above have approximately the same order of toxicity against fish as did the principle which Hauschild (4) obtained in a similar manner.

Ether Extract.—Several fractions from this extract proved to be very active inhibitors of uterine contractions *in vitro*. The first of these was a light brown amorphous substance, representing 0.0064% of the bark which was deposited on cooling the ether solution. This residue was found to be effective in a dilution of 1:10,000 in terms of the drug or 1:156,000,000 in terms of yield of residue.

After the removal of the above material, the ether solution was reduced to a volume of 150 cc. whereupon a dark brown resinous material was deposited, amounting to 0.0835% of the bark. Its alcoholic solution when tested pharmacologically showed effective sedation in concentrations as small as 1:167,000 of the drug or 1:208,332,000 of the material itself.

The residual ether solution yielded a dark green semisolid which was extracted with a minimum of 95% alcohol. The alcohol-soluble portion represented 0.7% of the bark. This fraction, although much less potent than the ether fractions mentioned before, still is an active inhibitor of the contractions of the isolated rat uterus. In a series of several experiments using this material, definite sedation was shown using a concentration of 1:50,000 of the drug or 1:7,142,000 of the extract itself.

Chloroform Extract.—The residue from this extract was a dark viscid material amounting to 0.317% of the bark. Although quite active as a uterine depressant, this fraction is not of the same order of potency as those previously reported for petroleum ether. In the few *in vitro* experiments made, concentrations of 1:35,000 of the drug or 1:11,000,000 of the extract produced uterine sedation.

An attempt to further separate the active principle of this fraction was made employing partially the methods used by Freer and Clover (8) on the chloroform extract in determining the identity of piscidin. The chloroform residue was redissolved in 50 cc. of chloroform and allowed to stand for several days and then filtered free of any deposit. The filtrate was then evaporated to 25 cc., diluted with an equal volume of ether, and then extracted with a 33% solution of KOH. The chloroform-ether solution was repeatedly washed with water until the latter became colorless, then with a trace of acid, finally dried over anhydrous sodium sulfate, and the ether-chloroform distilled. The separation was carried no further than this point. A reddish brown, partially crystalline material was obtained amounting to 0.037% of the bark. This proved to be the carrier of the active principle of this fraction, since the effective dilution in terms of bark was approximately the same as that of the chloroform extract from which it was derived. In terms of the material itself, it proved to be effective *in vitro* in dilutions up to 1:280,000,000. Cushny (14), however, in testing crystalline piscidin obtained in this manner, found it to be inactive. It is not known, however, if uterine studies were made. Pittenger and E'We (17), on the other hand, found it an effective hypnotic in dogs.

Absolute Methanol and 70% Ethanol Extracts.—Although quite active when compared to other botanicals used for this purpose, these extracts were not of the same order of potency as the petroleum ether and ether fractions. Upon being tested *in vitro*, the methanol extract was found to be effective in a concentration of 1:10,000 of the drug or 1:500,000 of the extract itself. Concentrations of 1:5000 of the 70% ethanol extract showed but questionable sedation.

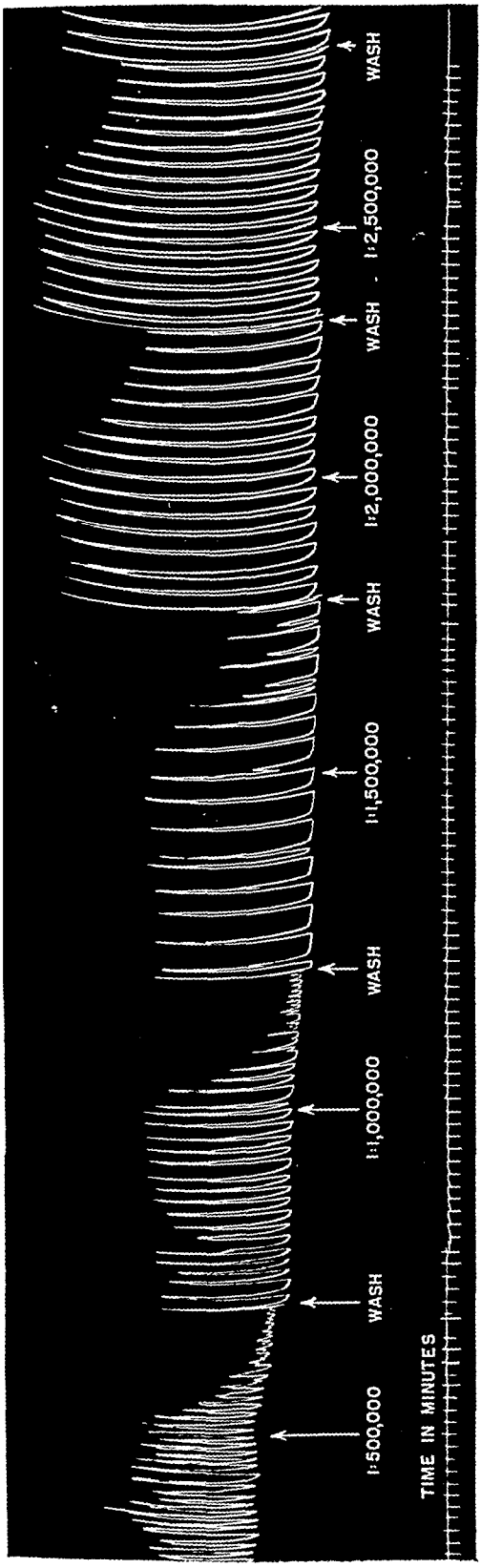
Determination of Optimum Solvent.—It has been shown that alcohol is by no means the optimum solvent for the uterine depressant principles of Piscidia. In order to determine the best single solvent for the active principles of the drug from a pharmaceutical standpoint, several of the other common solvents were tried on separate portions of commercial bark and the resultant dry extracts bioassayed. Table I illustrates the comparative merits of those employed.

TABLE I.—*Piscidia erythrina*. COMPARATIVE POTENCY *in Vitro* OF EXTRACTS OBTAINED WITH VARIOUS SOLVENTS

Solvent	Approx. Effective Dilution in Terms of Bark	Approx. Effective Dilution in Terms of Dry Extract	% of Drug Represented by Dry Extract	Method of Extraction
38.5% Ethanol[a]	1:20,000	1:265,000	7.53	Percolation
95% Ethanol	1:50,000	1:1,600,000	3.17	Soxhlet
60% Isopropanol	1:50,000	1:500,000	10.26	Percolation
Acetone	1:100,000	1:3,600,000	2.78	Soxhlet
Hot Water	1:111,000	1:488,000	22.73	Soxhlet
Chloroform	1:500,000	1:17,800,000	2.85	Soxhlet
Benzene	1:750,000	1:83,000,000	0.904	Soxhlet
Petroleum Ether[b]	1:1,000,000	1:260,000,000	0.373	Soxhlet

[a] Fluidextract.
[b] Alcohol-soluble portion.

Determination of Most Active Part of Plant.—Due to the contradictions in the literature as to the part used, and because past investigations have been limited to the stem and root barks, it was decided to investigate the comparative potencies of several parts of the plant. Having access to stands of the material in Florida and Jamaica, various parts of the



plant were collected for us which we submitted to bioassay. Portions of the various parts were refluxed with 95% alcohol containing calcium carbonate for over three hours and filtered hot. The residues were washed with hot alcohol and the washings added to the filtrates. Although a limited number of experiments were conducted, and different uteri varied in response, sufficient data were collected to demonstrate the comparative potency of the parts tested and to indicate that all these parts bear a potent principle depressant to the uterus *in vitro*. The part most potent appears to be the root bark which is effective in dilutions of from 1:1,000,000 to 1:3,000,000 (Fig. 6).

Table II illustrates these findings, expressed in average threshold dilutions in terms of part used.

TABLE II.—*Piscidia erythrina*. COMPARATIVE POTENCIES *in Vitro* OF VARIOUS PARTS OF THE PLANT

Part Used	Source	State	Effective Dilution
Leaves	Florida	Dried	1:100,000
	Jamaica	Dried	1:100,000
Young whole limbs	Jamaica	Dried	1:100,000
Young stem bark	Florida	Fresh	1:50,000
	Florida	Dried	1:200,000
Older stem bark	Jamaica	Dried	1:500,000
Young stem wood	Florida	Fresh	1:1,000,000
	Florida	Dried	1:500,000
Root wood	Florida	Fresh	1:500,000
	Florida	Dried	1:500,000
Root bark	Florida	Fresh	1:3,000,000
	Florida	Dried	1:1,000,000

On standing, crystalline material was deposited in the alcoholic extract of most of the above. This was separated and recrystallized, and a yield of white granular crystals, melting at 188°, was obtained amounting to 0.13% of the part used in the cases of the tree and root barks. The crystals had a sweet taste, were soluble in water and dilute alcohol, slightly soluble in 95% alcohol and absolute methanol, and insoluble in petroleum ether, ether, chloroform, acetone, and benzene. The principle reduced Fehling's solution only after hydrolysis with diluted hydrochloric acid at 100°. Upon bioassay, the principle was found to be inactive upon uterine tissue *in vitro* and *in vivo*, and no further chemical study was conducted.

Comparison of Uterine Sedative Effect (*in Vitro*).—It seemed a point of interest to us that a comparison be made of the inhibitory property of various standard preparations on uterine tissue and the sedative effect of certain Piscidia fractions. The experiments from which Table III was compiled were made in our laboratory and represent the activity of the various preparations as we have found

Fig. 6.—Effect on rat, 149 Gm. (anestrus), isolated uterus, right horn, *in vitro*. Drug: Piscidia, root bark extraction of dried drug, 95% alcohol.

TABLE III.—COMPARISON OF SEDATIVE DOSAGE OF PISCIDIA AND VARIOUS OTHER SMOOTH MUSCLE DEPRESSANTS (RAT UTERUS—*in Vitro*)

	Effective Sedative Dose (in Terms of Dried Drug)	Index of Potency (Viburnum Taken as 1)	Effective Sedative Dose (in Terms of Dried Extract)
1. Fluidextract *Viburnum prunifolium*	1:1000	1	.....
2. Fluidextract *Piscidia erythrina*	1:20,000	20	.....
3. Ethanol (95%) Extract, Piscidia	1:50,000	50	1:1,600,000
4. Acetone Extract, Piscidia	1:100,000	100	1:3,600,000
5. Benzene Extract, Piscidia	1:750,000	750	1:83,000,000
6. Ethanol-soluble portion of Petroleum Ether Extract, Piscidia	1:1,000,000	1000	1:260,000,000
7. Papaverine Hydrochloride	1:1,000,000	1000	.....
8. Ethanol Soxhlet of Root Bark, Piscidia	1:3,000,000	3000	.....

them. It will be noted that certain fractions of Piscidia compare favorably in action with Papaverine Hydrochloride which has been proposed as a standard (29, 30) for bioassay of uterine sedatives.

Toxicity.—In view of its history as a fish poison and hypnotic, a limited number of experiments were performed on laboratory animals and gold fish to ascertain the relative toxicity of Piscidia and some of its principles. These toxicity studies were not intended to be complete, but to serve as sighting experiments for further work. The fluidextract given orally *via* catheter to a group of twenty rats proved nontoxic at the dosage level of 20 Gm. of the drug per Kg. Symptoms such as grogginess and drowsiness could be explained partially by the hypnotic effect of the drug and the alcohol contained in the fluidextract. Control animals given the same volume of alcohol showed the same symptoms but in somewhat lesser degrees. All animals recovered within five to seven hours. Another series of rats was given a 60% isopropyl alcohol dried extract of Piscidia dispersed in water. Two out of a group of seven receiving 10,000 mg. per Kg. orally of the extract, equivalent to 106 Gm. of the dried bark, died within one hour, while the remaining five recovered. A group of ten rats receiving 7500 mg. per Kg. of the extract, equivalent to 80 Gm. of the dried bark, recovered within eight hours.

Hauschild (4) found the alcohol-soluble principle of the petroleum ether extract to be toxic to fish in dilutions of 1:80,000,000. In a series of tests employing thirty gold fish, we found the principle to be toxic in from 1:5,000,000 to 1:30,000,000. The lower toxic concentration may be explained in that larger fish were employed in our experiments.

SUMMARY

1. The present bark of commerce of Piscidia represents the stem bark, and not the root bark as reported by earlier investigators.

2. Preparations of Piscidia were found to be exceedingly potent uterine depressants *in vitro* and *in vivo* on various laboratory animals.

3. The drug was submitted to chemical separation in order to trace the depressant compounds. Such substances were found in the resin and the extracts of alcohol, petroleum ether, ether, and chloroform.

4. Petroleum ether is the optimum single solvent for the depressant principle.

5. All parts of the plant contain the depressant principle, the root bark being the most potent.

6. Certain fractions of the drug have the same order of uterine depression *in vitro* as Papaverine Hydrochloride and exceed any of the known botanicals used for this purpose in depressant action.

7. Acute studies indicate that Piscidia has a low relative toxicity when fed orally to rats. The work of Hauschild on the fish poisoning principle has been partially confirmed.

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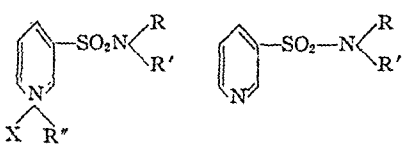
Quaternary Salts of N′-Substituted Pyridine-3-Sulfonamides*

By M. F. ZIENTY†

Methods for the preparation of several new N′-substituted pyridine-3-sulfonamides are described. From these eighteen new quaternary salts, several which possess germicidal activity were derived.

DOMAGK's demonstration (1) of the bactericidal properties of quaternary salts in which the nitrogen bears a long chain aliphatic substituent stimulated a number of investigations in this field (2–5).

A previous report from this laboratory (6) indicated that alkylpyridinium and alkyl picolinium halide salts possess conspicuous germicidal activity. The present communication describes the preparation and germicidal activity of a new class of quaternary salts having the general structure (I).



I II

R and R′ = alkyl radicals H, C_2H_5, C_8H_{17}, $C_{12}H_{25}$, $C_{14}H_{29}$, or $C_{16}H_{33}$
R″ = CH_3, C_7H_7, C_3H_5, $C_{12}H_{25}$, or $C_{16}H_{33}$
X = Cl, Br, I, or SO_4CH_3

Compounds of this class (I) were chosen in order to determine the efficacy of quaternary ammonium salts containing a long chain aliphatic substituent attached to the basic heterocyclic ring by other than a carbon-to-carbon bond or direct connection to the quaternary nitrogen.

Sulfonation of pyridine yielded pyridine-3-sulfonic acid (7, 8). After conversion to the corresponding sulfonyl chloride (7), the pyridine-3-sulfonamides (II) listed in Table I were prepared in yields of 63–77%.

The eighteen quaternary salts shown in Table II were obtained from II by one of the several methods described previously (6, 9). As indicated in this table, the germicidal activity of a number of these compounds compares favorably with the values reported for benzalkonium chloride (Zephiran) (10).

TABLE I.—N′-SUBSTITUTED PYRIDINE-3-SULFONAMIDES

Pyridine-3-sulfonamide	M. p., °C.	Formula	Nitrogen, % Calcd.	Nitrogen, % Found
N′-Dodecyl	67–68	$C_{17}H_{30}O_2N_2S$	8.61	8.75
N′-Tetradecyl	79–80	$C_{19}H_{34}O_2N_2S$	7.91	7.71
N′-Hexadecyl	78–79	$C_{21}H_{38}O_2N_2S$	7.32	7.07
N′-Di-2-ethylhexyl	Oil[a]	$C_{21}H_{38}O_2N_2S$	7.31	7.51
N′-Diethyl	46–47[b]	$C_9H_{14}O_2N_2S$	..	..

[a] B. p. 253–255°/16 mm.
[b] Machek, *Monatsh.*, 72, 87(1939); reported m. p. 49–50°.

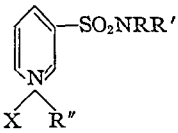
EXPERIMENTAL[1]

Pyridine-3-sulfonic acid.—To a mixture of 4.2 Gm. of mercuric sulfate and 700 Gm. of 30% oleum was added, cautiously, 120 Gm. (1.2 moles) of pyridine. The solution was heated under reflux at 300° (bath temperature) for eight hours. On cool-

* Received Oct. 3, 194[illegible] of The Upjohn Company.
† The author wishes [illegible] his valuable suggestions and interest in this work, and the Bacteriology Research Department of The Upjohn Company for the bacteriological data.

[1] All melting points are uncorrected. Microanalyses by Messrs. H. Emerson and W. Struck.

TABLE II.—QUATERNARY SALTS OF N-SUBSTITUTED PYRIDINE-3-SULFONAMIDES



R	R'	R''	X	M. p., °C.	Formula	Halogen, % Calcd.	Halogen, % Found	Activity [c]
$C_{12}H_{25}$	H	CH_3	I	96–97	$C_{18}H_{33}O_2N_2SI$	27.13	27.03	1–15,000
$C_{14}H_{29}$	H	CH_3	I	198–199	$C_{20}H_{37}O_2N_2SI$	25.60	26.91	1–20,000
$C_{16}H_{33}$	H	CH_3	I	165–166	$C_{22}H_{41}O_2N_2SI$	24.24	24.41	1–10,000
C_8H_{17} [a]	C_8H_{17} [a]	CH_3	I	144–145	$C_{22}H_{41}O_2N_2SI$	24.24	24.02	1–35,000
$C_{12}H_{25}$	H	C_7H_7	Cl	157–158	$C_{24}H_{37}O_2N_2SCl$	7.74	7.71	1–30,000
$C_{14}H_{29}$	H	C_7H_7	Cl	160–161	$C_{26}H_{41}O_2N_2SCl$	7.36	7.16	1–10,000
$C_{16}H_{33}$	H	C_7H_7	Cl	160–161	$C_{28}H_{45}O_2N_2SCl$	6.89	6.86	1–25,000
C_8H_{17} [a]	C_8H_{17} [a]	C_7H_7	Cl	Oil	$C_{28}H_{45}O_2N_2SCl$	6.89	6.82	1–20,000
$C_{12}H_{25}$	H	C_3H_5	Br	102–103	$C_{28}H_{35}O_2N_2SBr$	17.89	17.76	1–10,000
$C_{14}H_{29}$	H	C_3H_5	Br	109–110	$C_{22}H_{39}O_2N_2SBr$	16.84	16.95	1–10,000
$C_{16}H_{33}$	H	C_3H_5	Br	105–106	$C_{24}H_{43}O_2N_2SBr$	15.90	15.70	1–25,000
C_8H_{17} [a]	C_8H_{17} [a]	C_3H_5	Br	74–75	$C_{24}H_{43}O_2N_2SBr$	15.90	15.88	1–25,000
$C_{12}H_{25}$	H	CH_3	SO_4CH_3	Oil	$C_{19}H_{36}O_6N_2S_2$	(6.19) [b]	(6.21) [b]	1–1,000
$C_{14}H_{29}$	H	CH_3	SO_4CH_3	110–111	$C_{21}H_{40}O_6N_2S_2$	(6.25) [b]	(6.14) [b]	1–1,000
$C_{16}H_{33}$	H	CH_3	SO_4CH_3	104–105	$C_{23}H_{44}O_6N_2S_2$	(5.51) [b]	(5.77) [b]	1–1,000
C_8H_{17} [a]	C_8H_{17} [a]	CH_3	SO_4CH_3	Oil	$C_{23}H_{44}O_6N_2S_2$	(5.51) [b]	(5.72) [b]	1–10,000
C_2H_5	C_2H_5	$C_{12}H_{25}$	Br	95–97	$C_{21}H_{39}O_2N_2SBr$	17.31	17.56	1–1,000
C_2H_5	C_2H_5	$C_{16}H_{33}$	Br	77–78	$C_{25}H_{47}O_2N_2SBr$	15.41	15.74	1–1,000

[a] 2-Ethylhexyl.
[b] Nitrogen, %.
[c] Determined by the F.D.A. method, using *Staphylococcus aureus* at 37°, described in Circular 198 of the U. S. Department of Agriculture.

ing, the reaction mixture was poured into 1 L. of cracked ice and water. The solution was neutralized with solid barium carbonate and the precipitated barium sulfate removed by suction filtration. The filter cake was washed well with hot water to remove all the sulfonic acid. The filtrate and washings were combined and evaporated nearly to dryness. Treatment of the residue with concentrated sulfuric acid followed by filtration to remove barium sulfate and concentration of the filtrate yielded 131 Gm. (55%) of colorless needles, m. p. 365–366°.

Pyridine-3-sulfonyl chloride.—Ten grams (0.078 mole) of pyridine-3-sulfonic acid was triturated in a mortar with 13.2 Gm. of phosphorus pentachloride, transferred to a ground-glass jointed flask and heated under reflux at 160–165° until the evolution of hydrogen chloride has ceased (two to three hours). The flask was fitted with an inclined condenser and the phosphorus oxychloride removed under reduced pressure. The residual yellowish oil was the crude pyridine-3-sulfonyl chloride which was used in subsequent reactions without further purification.

Pyridine-3-N'-alkylsulfonamides.—The N'-alkylsulfonamides listed in Table I were prepared by the procedure used to synthesize pyridine-3-N'-hexadecylsulfonamide.

Pyridine-3-N'-hexadecylsulfonamide.—A solution of pyridine-3-sulfonyl chloride in 50 cc. of dry benzene, prepared from 10 Gm. (0.078 mole) of pyridine-3-sulfonic acid, was well cooled in ice and treated with a solution of 30 Gm. (0.12 mole) of hexadecylamine in 100 cc. of dry benzene. The copious precipitate of amine hydrochloride formed was removed by suction filtration and the filtrate evaporated to dryness. The solid residue was crystallized from a mixture of benzene and petroleum ether (90–100°), and yielded 15 Gm. (63%) of colorless crystals, m. p. 78–79°.

Anal.—Calcd. for $C_{21}H_{38}N_2O_2S$: N, 7.32. Found: N, 7.07.

Pyridine-3-N'-hexadecylsulfonamide Methiodide.—A solution of 1.25 Gm. (0.0033 mole) of pyridine-3-N'-hexadecylsulfonamide and 0.60 Gm. (0.0042 mole) of methyl iodide in 15 cc. of absolute alcohol, was refluxed for six hours. The solvent was removed by evaporation and the yellow solid dissolved in ethyl acetate. On cooling, 1.5 Gm. (78%) of a light yellow amorphous solid was obtained, m. p. 165–166°.

Anal.—Calcd. for $C_{22}H_{40}N_2O_2SI$: I, 24.28. Found: I, 24.41.

Pyridine-3-N'-hexadecylsulfonamide Benzylchloride.—A mixture of 1.5 Gm. (0.004 mole) of pyridine-3-N'-hexadecylsulfonamide and 0.49 Gm. (0.004 mole) of benzyl chloride was heated in an oil bath at 110° for two hours. On cooling, a solid crystalline mass was obtained which was recrystallized from ethyl acetate to yield 1.8 Gm. (90%) of lustrous plates, m. p. 160–161°.

Anal.—Calcd. for $C_{29}H_{45}N_2O_2SCl$: Cl, 6.97. Found: Cl, 6.86.

Pyridine-3-N'-hexadecylsulfonamide Allylobromide.—One gram (0.0026 mole) of pyridine-3-N'-hexadecylsulfonamide and 0.35 Gm. (0.003 mole) of allyl bromide was heated under reflux for ten minutes. The heating was interrupted, the flask stoppered and allowed to stand at room temperature overnight. The crystalline mass was dissolved in

acetone and skelly-solv "B" added until the solution became slightly turbid. On standing, 1 Gm. (80%) of nearly colorless plates was obtained, m. p. 105–106°.

Anal.—Calcd. for $C_{24}H_{43}N_2O_2SBr$: Br, 15.90. Found: Br, 15.70.

Pyridine-3-N′-hexadecylsulfonamide Methosulfate.—A mixture of 2 Gm. (0.0052 mole) of pyridine-3-N′-hexadecylsulfonamide and 0.75 Gm. (0.006 mole) of dimethyl sulfate was heated on a steam cone for three hours. The unreacted dimethyl sulfate was removed by washing with petroleum ether. The semisolid was dissolved in ethyl acetate, decolorized, and filtered. Colorless plates were obtained; yield 2.1 Gm. (80%), m. p. 104–105°.

Anal.—Calcd. for $C_{23}H_{44}N_2O_6S_2$: N, 5.51. Found: N, 5.77.

Pyridine-3-N′-diethylsulfonamide Laurylobromide.—Ten grams (0.047 mole) of pyridine-3-N′-diethylsulfonamide was heated with 11.7 Gm. (0.047 mole) of lauryl bromide at 120° for two hours. Recrystallization of the solid from a mixture of petroleum ether and dry ether yielded 18 Gm. (83%) of colorless crystals, m. p. 95–97°.

Anal.—Calcd. for $C_{23}H_{43}N_2O_2SBr$: Br, 17.31. Found: Br, 17.56.

SUMMARY

Details are given for the preparation of several new N′-substituted pyridine-3-sulfonamides and, from these, eighteen new quaternary salts. The germicidal activity of several of the new quaternary salts has been shown to compare favorably with other established germicides.

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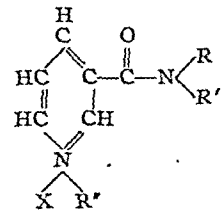
Quaternary Salts of Pyridine-3-Carboxamides*

By M. F. ZIENTY†

The preparation of twenty-seven salts of nicotinamide, N′-N′-diethylnicotinamide and 4-(nicotinyl) morpholine, is reported. Each of these compounds were found to be active germicides.

THE high germicidal activity of a number of pyridinium, 2-picolinium and 4-picolinium salts (1) suggested that other quaternary salts of substituted pyridines be prepared and evaluated.

A survey of the literature disclosed that several quaternary salts of nicotinamide (2) and the methiodide and methochloride of nikethamide (3), but no quaternary salts of 4-(nicotinyl)-morpholine, had been prepared. However, none of these had been tested for germicidal activity. Therefore it seemed desirable to prepare a series of quaternary salts of these amides and explore the possibilities of this class of compounds having the general structure (I).



I

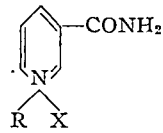
R and R′ = H, C_2H_5 or $—CH_2—CH_2—O—CH_2—CH_2—$
R″ = $C_{12}H_{25}$, $C_{14}H_{29}$, $C_{16}H_{33}$, or $C_{18}H_{37}$
X = Cl, Br, or I

The quaternary salts of nicotinamide were prepared by heating equivalent amounts of amide with an alkyl halide at 150–155° for eight hours.

The reaction between 4-(nicotinyl)-morpholine or nikethamide and an alkyl halide, without a solvent, gave only dark-

* Received [illegible] of The Upjohn [illegible]
† The aut[illegible] his advice and suggestions in this work, and the Bacteriology Research Department of The Upjohn Company for the bacteriological data.

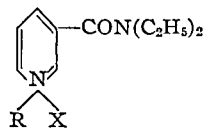
TABLE I.—ALKYL-3-CARBAMYLPYRIDINIUM HALIDES



R	X	M. p., °C.	Formula	Nitrogen, % Calcd.	Nitrogen, % Found	Activity
$C_{12}H_{25}$	Cl	237–239	$C_{18}H_{31}N_2OCl$	8.58	8.55	1–5,000
$C_{14}H_{29}$	Cl	246–247	$C_{20}H_{35}N_2OCl$	(9.88)[a]	(9.02)[a]	1–15,000
$C_{16}H_{33}$	Cl	245–246[b]	$C_{22}H_{39}N_2OCl$	7.33	7.56	1–15,000
$C_{12}H_{25}$	Br	229–230	$C_{18}H_{31}N_2OBr$	7.54	7.42	1–5,000
$C_{14}H_{29}$	Br	229–230	$C_{20}H_{35}N_2OBr$	7.02	6.98	1–15,000
$C_{16}H_{33}$	Br	225–226	$C_{22}H_{39}N_2OBr$	6.55	6.49	1–15,000
$C_{18}H_{37}$	Br	224–225	$C_{24}H_{43}N_2OBr$	4.50	4.79	1–2,000
$C_{12}H_{25}$	I	177–178	$C_{18}H_{31}N_2OI$	(30.30)[a]	(29.80)[a]	1–10,000
$C_{14}H_{29}$	I	176–178	$C_{20}H_{35}N_2OI$	(28.42)[a]	(29.62)[a]	1–5,000
$C_{16}H_{33}$	I	175–177	$C_{22}H_{39}N_2OI$	5.90	5.52	1–5,000
$C_{18}H_{37}$	I	173–175	$C_{24}H_{43}N_2OI$	5.57	5.48	1–2,000

[a] Halogen, %.
[b] Karrer and Stare, *Helv. Chim. Acta*, **20**, 423(1937), reported m. p. 235°.

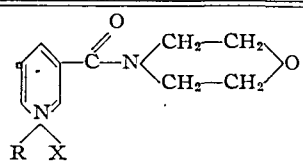
TABLE II.—ALKYL-3-DIETHYLCARBAMYLPYRIDINIUM HALIDES



R	X	M. p., °C.	Formula	Nitrogen, % Calcd.	Nitrogen, % Found	Activity
$C_{12}H_{25}$	Br	Oil	$C_{22}H_{39}N_2OBr$	6.55	6.79	1–5,000
$C_{14}H_{29}$	Br	57–58	$C_{24}H_{43}N_2OBr$	6.15	6.28	1–10,000
$C_{16}H_{33}$	Br	63–64	$C_{26}H_{47}N_2OBr$	5.79	5.88	1–10,000
$C_{18}H_{37}$	Br	68–69	$C_{28}H_{51}N_2OBr$	(15.63)[a]	(14.34)[a]	1–5,000
$C_{12}H_{25}$	I	Oil	$C_{22}H_{39}N_2OI$	5.90	5.84	1–5,000
$C_{14}H_{29}$	I	Oil	$C_{24}H_{43}N_2OI$	5.57	5.73	1–5,000
$C_{16}H_{33}$	I	52–55	$C_{26}H_{47}N_2OI$	5.28	5.06	1–10,000
$C_{18}H_{37}$	I	62–63	$C_{28}H_{51}N_2OI$	(22.79)[a]	(23.05)[a]	1–5,000

[a] Halogen, %.

TABLE III.—QUATERNARY SALTS OF 4-(NICOTINYL)-MORPHOLINE



R	X	M. p., °C.	Formula	Nitrogen, % Calcd.	Nitrogen, % Found	Activity
$C_{12}H_{25}$	Br	54–55	$C_{22}H_{37}N_2O_2Br$	6.34	6.55	1–2,000
$C_{14}H_{29}$	Br	83–85	$C_{24}H_{41}N_2O_2Br$	5.97	6.14	1–10,000
$C_{16}H_{33}$	Br	74–75	$C_{26}H_{45}N_2O_2Br$	5.63	5.82	1–10,000
$C_{18}H_{37}$	Br	78–79	$C_{28}H_{49}N_2O_2Br$	5.33	5.05	1–2,000
$C_{12}H_{25}$	I	144–145	$C_{22}H_{37}N_2O_2I$	5 74	5.90	1–2,000
$C_{14}H_{29}$	I	145–146	$C_{24}H_{41}N_2O_2I$	5.42	5.55	1–5,000
$C_{16}H_{33}$	I	152–153	$C_{26}H_{45}N_2O_2I$	5.30	5.39	1–2,000
$C_{18}H_{37}$	I	153–154	$C_{28}H_{49}N_2O_2I$	(22.20)[a]	(22.52)[a]	1–2,000

[a] Halogen, %.

colored tars. However, if the reactants were dissolved in dry xylene and refluxed for eight hours, colorless, crystalline products were obtained. Repeated attempts to react either 4-(nicotinyl)-morpholine or nikethamide with alkyl chlorides failed. The unreacted starting materials were always recovered. Nicotinamide used in this work was the U. S. P. XII product, available commercially, while the nikethamide and 4-(nicotinyl)-morpholine were prepared, essentially, according to directions in the literature (4).

Germicidal tests were made according to the F.D.A. method using *Staphylococcus aureus* at 37° (5).

The compounds were active as germicides. Without exception, the salts of the amides were less active than the corresponding pyridinium or picolinium compounds (1). The most active compounds were the quaternary salts of nicotinamide, those of nikethamide next, and those of 4-(nicotinyl)-morpholine the least active. The solubilities of these compounds, in aqueous solutions, were in the reverse order.

EXPERIMENTAL[1]

4-(Nicotinyl)-morpholine.—A mixture of 92 Gm. (1.05 moles) of morpholine and 123 Gm. (1 mole) of nicotinic acid was cooled in ice, cautiously treated with 77 Gm. (0.5-mole) of phosphorous oxychloride, and the solution heated on a steam bath, under reflux, for ten hours. The resulting semisolid reaction mixture was dissolved in 400 cc. of water and the solution saturated with solid potassium carbonate. The upper oily layer was separated and dried under reduced pressure at the temperature of the steam bath. The nearly solid slurry was treated with 400 cc. of dry benzene and the inorganic salts removed by suction filtration. Removal of the benzene under reduced pressure gave an oil which boiled at 196–200°/10 mm.,[2] yielding 69 Gm. (35%) of a heavy yellow oil.

N′,N′-Diethylnicotinamide.—A three-liter, three-necked flask, fitted with a stirrer, reflux condenser, and dropping funnel was charged with 492 Gm. (4 moles) of nicotinic acid. The stirrer was started and 308 Gm. (4.2 moles) of diethylamine allowed to run into the flask, with external cooling. Then 608 Gm. of phosphorous oxychloride was slowly dropped into the mixture. The stirring was interrupted and the dark solution heated on a steam bath overnight. The thick, sirupy liquid was poured into a mixture of cracked ice and sodium hydroxide solution (500 Gm. sodium hydroxide in 500 cc. of water). The upper layer was separated and the lower aqueous layer extracted with four 200-cc. portions of benzene. The organic layer and extracts were combined and a small amount of water separated. Removal of the benzene under reduced pressure gave a dark oil which was distilled to yield 498 Gm. (70%) of nearly colorless product; b. p. 163–168°/11 mm.

Lauryl-3-carbamylpyridinium Bromide.—A mixture of 12.2 Gm. (0.1 mole) of nicotinamide and 25 Gm. (0.1 mole) of lauryl bromide was heated in a metal bath, so that an internal temperature of 150–155° was maintained, for eight hours. The mixture was cooled and the solid dissolved in hot ethanol, decolorized with Darco and filtered. On cooling, 26 Gm. (70%) of yellowish crystals were obtained. Several crystallizations yielded colorless material, m. p. 227–228°.

Anal.—Calcd. for $C_{18}H_{31}N_2OBr$: N, 7.54. Found: N, 7.22.

Dodecyl-4-(nicotinyl)-morpholine Iodide.—A solution of 10 Gm. (0.05 mole) 4-(nicotinyl)-morpholine and 14.8 Gm. (0.05 mole) of dodecyl iodide in 40 cc. of dry xylene was heated under reflux for eight hours. The solvent was removed and the semisolid, obtained on cooling, rubbed under ethyl acetate until crystallization took place. Two crystallizations from ethyl acetate yielded 11 Gm. (50%) of lemon-colored crystals, m. p. 144–145°.

Anal.—Calcd. for $C_{22}H_{37}N_2O_2I$: N, 5.74. Found: N, 5.90.

Hexadecyl-3-diethylcarbamylpyridinium Bromide.—A solution of 10 Gm. (0.056 mole) of nikethamide and 17 Gm. (0.056 mole) of hexadecyl bromide in 40 cc. of dry xylene was refluxed for eight hours The xylene was removed and the semisolid mass, obtained on cooling, dissolved in ethyl acetate and a small amount of ethanol. On cooling, 8 Gm. (30%) of colorless crystals were obtained; m. p. 63–64°.

Anal.—Calcd. for $C_{26}H_{47}N_2OBr$: N, 5.79 Found: N, 5.88.

SUMMARY

A series of twenty-seven salts of nicotinamide, N′,N′-diethylnicotinamide and 4-(nicotinyl) morpholine has been prepared.

The compounds are active germicides.

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(4) French Patent 824,042(1938); *C. A.*, 32, 4285(1938).
(5) Rue le, G. L. A., and Brewer, C. M., U. S. Dept. Agr. Circ., No. 198, 4(1931).

[1] All melting points are uncorrected. Microanalyses by Messrs. H. Emerson and W. Struck and Misses Celia Triemstra and Barbara Fausnaugh.

[2] French Patent 824,042(1938); *C. A.*, 32, 4285(1938); reported b. p. 210°/15 mm.

Synthesis of Tetraiodinated Congo Red*†‡

By CHARLES F. MARTIN§ and HAAKON BANG‖

3,3′,5,5′-Tetraiodobenzidine and tetraiodinated Congo red were prepared. The compounds were analyzed for their iodine content and their solubilities were determined. Pharmacological and radiological studies of tetraiodinated Congo red have thus far indicated that it is nontoxic, but that it is not concentrated in amyloid tissue in sufficient quantity to produce any opacity with X-ray. Further tests of this type are anticipated.

THIS report is based on a part of the work undertaken by the authors in the search for new iodinated compounds which might find use as radiographic opaque agents. Pharmacological and radiological studies have indicated, thus far, that tetraiodinated Congo red is no more toxic than Congo red, but that, after administration, the concentration of it in amyloid tissue is not great enough to cast a shadow on the X-ray plate (1). Further pharmacological and radiological studies of the compound are anticipated.

EXPERIMENTAL

3,3′,5,5′-Tetraiodobenzidine.—Nineteen and two-tenth grams of benzidine dihydrochloride was dissolved, by the aid of heat and constant mechanical stirring, in 1200 cc. of distilled water containing 184 cc. of concentrated hydrochloric acid. When solution was complete, stirring was continued and the temperature was adjusted to 80°. At this time, 1500 cc. of a solution containing 75 Gm. of iodine and 150 Gm. of potassium iodide was added in 100-cc. portions during a period of about thirty minutes. Stirring was continued and the temperature was maintained at 80° for two hours after the last of the iodine solution had been added.

The dark red solution was removed from the heat and allowed to cool to room temperature. The crystals, thus obtained, were collected, dried in air, and washed with three 200-cc. portions of cold carbon tetrachloride. The product was then dried in air and analyzed for its iodine content by the method used by Klemme and Hunter (2).

Anal.—Calcd. for $C_{12}H_8I_4N_2$: I, 73.8%. Found: I, 73.3% and 73.1%.

The product is purple-colored needles which will not melt when heated to 300°. It is insoluble in water, carbon tetrachloride, chloroform, petroleum ether, benzene and acetic acid. It is soluble in ethanol, methanol, acetone and ether. The yield was 98%.

Tetraiodinated Congo Red.—Forty and eight tenths grams of finely powdered 3,3′,5,5′-tetraiodobenzidine was suspended in 300 cc. of 10% hydrochloric acid. This mixture was cooled in an ice-salt bath to 0° with constant stirring. A solution containing 12.6 Gm. of sodium nitrite in 15 cc. of cold distilled water was added in small portions to the mixture. Stirring was continued at the lowered temperature for thirty minutes after all of the sodium nitrite solution had been added.

The cold mixture containing the tetrazotized compound was poured, in small portions, into a stirred solution containing 30.6 Gm. of sodium naphthionate and 90 Gm. of sodium carbonate in 1200 cc. of cold distilled water. When effervescence had subsided, the dark-red, alkaline solution was heated to boiling and allowed to stand overnight. The solution was filtered and the filtrate was evaporated to dryness on a water bath. The residue was powdered in a mortar and extracted with a liter of absolute methanol the first time, and then with three 400-cc. portions of the same solvent.

The methanol extracts were combined, filtered, and evaporated to dryness on a water bath. The residue was dried in an oven at 100°, powdered, and extracted once with 1200 cc. of absolute ethanol.

The ethanol extract was filtered and evaporated to dryness. The residue was powdered and then washed with a solution containing three parts of ether and one part of absolute ethanol. The dye was dried in an oven at 100° and then analyzed for its iodine content by the method used before.

Anal.—Calcd. for $C_{32}H_{18}I_4N_6Na_2O_6S_2$: I, 42.3%. Found: I, 42.8% and 42.7%.

The dye is a dark red powder, insoluble in ether, chloroform, acetone, petroleum ether and benzene. It is soluble in ethanol and methanol. One gram is soluble in about 2.4 Gm. of water. The yield was 51.8%.

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† This paper is based on a portion of a thesis to be submitted to the Graduate Studies Committee of the State College of Washington by Charles F. Martin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
‡ This investigation was supported by a grant from Lederle Laboratories, Inc., Pearl River, New York.
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Pharmacognostical Studies of Punarnavā

Stem and Leaf Characteristics of *Boerhaavia diffusa* Linn. and *Trianthema Portulacastrum* Linn.*

By S. PRASAD†

Punarnavā has been employed in India from earliest times as a specific against edema, beriberi, anemia, and heart diseases. The pharmacognostical characteristics of this drug are described and illustrated.

THERE exists some confusion with regard to the correct identity of the drug, Punarnavā, which has been employed in the Ayurvedic system of medicine as a specific against edema or dropsical conditions, beriberi, anemia, heart diseases, and also as a rejuvenant. Two varieties of this drug, namely, "Shwet Punarnavā" or white-flowered variety and "Rakta Punarnavā" or red-flowered variety, have been mentioned in the Ayurved. The plants corresponding to these have been identified as *Trianthema Portulacastrum* Linn. (*T. monogyna* Linn.) and *Boerhaavia diffusa* Linn. (*B. repens* Linn.), respectively [*cf.* Chakravarty (1)]. Sircar (2), however, is of the opinion that *Boerhaavia diffusa* which has been described to be Punarnavā by previous workers (3–6), is a different drug, "Kathillaka" by name, having similar therapeutic properties but being a little less potent than the real Punarnavā. According to him, *Trianthema Portulacastrum* is in fact Punarnavā, inasmuch as this plant possesses two kinds of flowers, one white and the other slightly red or pinkish; the white-flowered type thus represents "Shwet Punarnavā" and the other type as "Rakta Punarnavā."

The chemical and pharmacological studies of the above-mentioned species have been made by many investigators. Ghoshal (7) isolated from *Boerhaavia diffusa* an alkaloid, an oily mass of the nature of fat, sulfates and chlorides, and traces of nitrates and chlorates. Chopra, *et al.* (8) obtained sufficient amount of potassium nitrate and also an alkaloid which they named as "Punarnavine." Later Chopra, Chatterji, and Ghosh (9) found that the alkaloid "Punarnavine" and potassium nitrate were present in both the species, but that the percentages of these substances were greater in the red variety of *Trianthema Portulacastrum* than either in the white variety of this species or in *Boerhaavia diffusa*. Pharmacologically, too, these species have been found to possess more or less similar properties, producing a marked diuresis in cases of edema and ascites.

The therapeutic and chemical alliedness and also the close external resemblance between these two species appear to have been the main cause of confusion in the correct identity of Punarnavā. While many Indian pharmaceutical concerns have employed *Boerhaavia diffusa* for preparations of liquid extracts, etc., of Punarnavā, it is also not uncommon to get samples of *Trianthema Portulacastrum* from drug dealers when Punarnava is required. It was, therefore, decided to investigate the pharmacognostical characteristics of these plants, since no such study has yet been made in a systematic way, in order that these may be distinguished from each other. It may be pointed out, however, that some anatomical observations have already been made on the leaves of *Boerhaavia diffusa* by Sabnis (10), Dastur and Saxton (11), and Bhargava (12), but no detailed account has been given by any of them. So far as the stem anatomy of this species is concerned, the earlier account by Sabnis (10) is very brief. A detailed anatomical account of the stem was, however, published by Maheshwari (13), but the pharmacognostical characteristics were not indicated by him. Furthermore, no study on *Trianthema Portulacastrum* has yet been brought on record. The present investigation, dealing with the pharmacognostical studies of the stem and the leaf, including

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the stomatal index and palisade ratio of both these species, was therefore undertaken.

MATERIALS AND METHODS

Fresh specimens of *Boerhaavia diffusa* and *Trianthema Portulacastrum* were collected from different localities in the Benares Hindu University Campus. Free hand sections were taken from the different portions of leaf and stem and stained in safranin and gentian violet and mounted in Canada balsam in the usual way. The palisade ratio and the stomatal index were determined as described elsewhere (14). To ascertain if the values pertaining to palisade ratio and stomatal index were significantly different from species to species or from locality to locality, the data were statistically analyzed.

EXPERIMENTAL

External Morphology

Boerhaavia diffusa.—It is a diffusely branched, nonsucculent herb, distributed all over India and ascending to 7000 ft. altitude in the warm valleys of the Himalayas. It is found also in Ceylon, Malaya Peninsula, extending to Australia, China, Africa, and America.

The root is stout and fusiform with a woody rootstock. There arise from the crown of the root, numerous stems 2–3 ft. long or even more, slender, prostrate or ascending, swollen at the nodes, and covered with numerous hairs. The leaves are rather thick, opposite, arising in unequal pairs at each node, the larger one being 1–1 1/2 by 3/4–1 inch and the smaller one 1/2–3/4 inch by 1/2 inch. They are broadly ovate, oblong or suborbicular with slightly pointed or rounded apex, and subcordate and rounded base. The margin is entire and subundulate, being turned up and pinkish in certain cases. The upper surface is green and glabrous, but the lower is slightly white. Very often white patches are seen on both surfaces. The petiole is nearly as long as the blade.

The flowers are very small, pedicellate, 4–10 grouped together in small bracteolate umbells, forming slender, long-stalked axillary and terminal panicles. Bracteoles are lanceolate and acute. The perianth tube is about 1/8 inch long, ovoid below, funnel-shaped in the limb, five-lobed, and deep pink in color. The lower part of the tube is strongly ribbed (Fig. 1, *A*). Stamens are 2–3, but sometimes only one and slightly exserted. Ovary is free, strongly stipitate and unilocular with a single basal, erect ovule. It is provided at the top with a filiform style having a peltate stigma. The fruit is 1/8 inch long, clavate, rounded, viscidly glandular on the blunt ribs (Fig. 1, *C*).

Trianthema Portulacastrum.—It is a prostrate, branched, and somewhat succulent herb which grows profusely in the plains with the advent of rains and dies out practically by winter. It is distributed throughout tropical India and Ceylon.

The stem is more or less angular, glabrous or pubescent, and much branched. The leaves are subfleshy, arranged obliquely opposite, in unequal pairs, the upper one being larger about 3/4–1 1/2 inch by 3/4–1 1/4 inch and the lower one 3/8–1/2 inch by 1/4–3/4 inch. They are broadly abovate, rounded, and often apiculate at the apex, cuneate at the base and glabrous. The petiole is 1/4–1/2 inch long, very much dilated and membranous at the base, especially in smaller leaves. These membranous enlargements form a triangular pouch and contain sessile, solitary flowers, fruits, and seeds. Calyx segments are ovate, acute, and calyx tube scarious, thin, closely sheathed by the base of the petiole. Stamens are 10–20. Ovary is obliquely truncate. Style one. Capsule is small, almost concealed in the petiolar pouch, lid truncate, slightly concealed with 2 spreading teeth, carrying away at least 1 seed, and the lower part 2–5 seeded. Seeds are reniform, dull, black, and muricate (Fig. 2, *B*, *C*).

Microscopical Features

Boerhaavia diffusa

Stem.—A transverse section of the young stem shows on the outside a single layer of epidermis, covered externally with cuticle and surrounded by numerous multicellular hairs of the glandular type. There is a deposition of crystalline granules on the epidermal walls below the cuticle. The hairs are uniseriate and consist of a stalk of 8–12 cells and an ellipsoidal or clavate head. The total length of a single hair is 150–220 microns.

The epidermal cells are cubical, measuring 17–20–25 microns by 20–25–32 microns.[1] Below the epidermis is a broad region of cortex consisting of 1–2 layers of collenchyma and 6–7 layers of parenchyma (Figs. 19–22). The cells of collenchyma are present

[1] The measurements of cells and other structures are given ordinarily in three separate figures, of which the first and the third represent the minimum and the maximum values respectively, while the middle indicates the more common value.

Legend to Figures

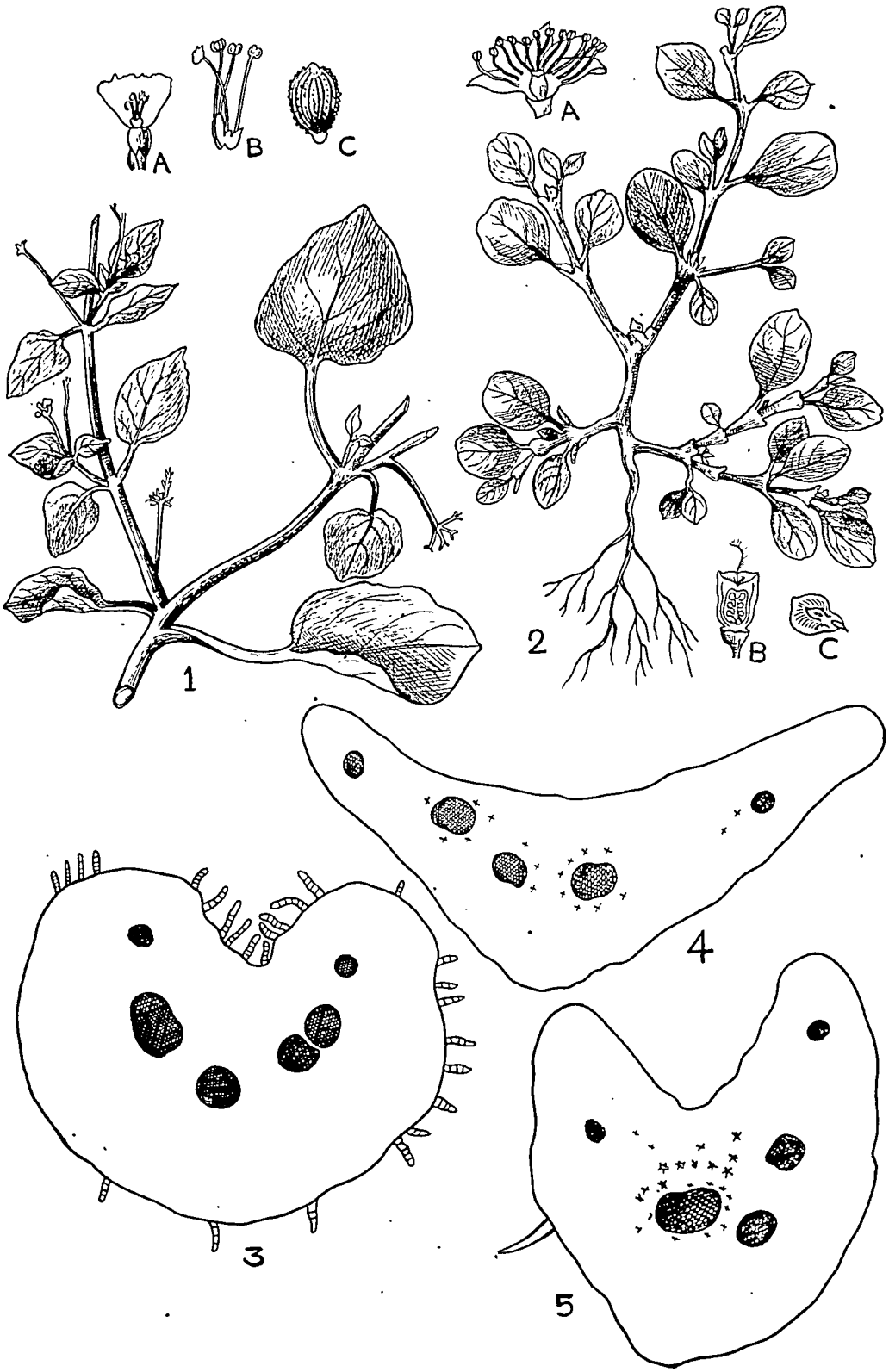
Fig 1.—A part of the plant. *Boerhaavia diffusa.* ×4/5. *A*—Flower dissected. ×13. *B*—Inflorescence. ×1 1/4. *C*—Fruit. ×13.

Fig. 2.—The whole plant of *Trianthema Portulacastrum.* ×1/2. *A*—Flower dissected. ×13. *B*—Ovary cut longitudinally. ×20. *C*—Seed cut longitudinally. ×25.

Fig. 3.—Transverse section of the petiole of *Boerhaavia diffusa.* ×40.

Fig. 4.—Transverse section of the basal part of the petiole of *Trianthema Portulacastrum.* ×40.

Fig. 5.—Transverse section of the upper part of the petiole of the same plant. ×40.


A
B
C
A
1
2
B
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4
5

more at the angles of the stem and are smaller in dimensions than those of the parenchyma. They measure 20–28 microns by 10–20 microns in transverse section. The parenchymatous cells are more or less isodiametric, measuring 20–34–40 microns in diameter. The endodermis is very indistinct, not easily to be made out. Following the endodermis is a thin strip of pericycle of 1–2 layers of cells, surrounding the stele. Often a few fibers may be found scattered beneath the endodermis. The stele is composed of two large bundles in the center, surrounded by a ring of 6–12 loosely arranged bundles and another outer ring of very small bundles, 15–20 or sometimes more in number (Fig. 19). The arrangement of the bundles thus gives the stem the appearance of a monocot stem, except for the presence of intrafascicular cambium.

The two central bundles are the largest in the stem and have a tangential diameter about twice as long as the radial. The secondary xylem elements produced by the intra-fascicular cambium of these bundles, toward the inner side, are arranged in regular radial rows. The secondary phloem produced toward the outside represents the phloem elements of these bundles, since the primary phloem gets crushed very soon. The secondary growth in the middle ring of vascular bundles is also very limited and the primary phloem, like that of the central bundle, gets crushed by the secondary phloem forming below [Maheshwari (13)]. The intrafascicular cambium of the outer ring is belated in its development and joins up to produce a complete cambium cylinder which gives rise later to a broad zone of anomalous wood (Figs. 20 and 21). The cambium cells measure 10–14 microns in tangential length and 5–7 microns in radial width and are 100–125 microns long. The secondary tissue produced by the cambium ring, consists of secondary xylem and secondary phloem in the intra-fascicular region and usually of conjunctive tissue and storage parenchyma in the inter-fascicular region (Fig. 22). The xylem vessels show bordered pits on their walls and measure 17–20–34 microns in diameter in the transverse section. The secondary phloem consists of sieve tubes, companion cells and phloem parenchyma. The sieve tubes measure 110–140 microns in length and 6–1 0 microns in breadth.

The inter-fascicular cambium forms on the inner side, fibers which are similar to substitute fibers, elongated and living and contain starch in them. They measure 10–14–20 microns in diameter and are lignified. The cells formed on the outer side, on the other hand, are thin-walled parenchyma arranged in radial rows. These are much bigger and almost isodiametric in transverse section.

While the secondary tissues are differentiating and their inner products becoming lignified, the outer cells of the pith lying in immediate contact with the lignified tissues of the vascular ring, also become lignified and very hard. The inner cells of the pith, however, remain parenchymatous and measure 50–68–85 microns in diameter.

While the activity of the first cambium begins to decline, additional cambium rings arise by the renewed cambial activity in the outer parenchymatous cells, produced by the first cambium. Outside the primary phloem, this new cambium passes through the cells of the pericycle. The new cambium thus formed functions only for some time and is replaced by another cambium by a similar process, which may be repeated until four or five or more growth rings are produced (Fig. 21). The mode of secondary growth is similar in all the supernumerary cambiums. The secondary xylem and phloem are always found opposite each other so as to give rise to true collateral bundles.

Petiole.—The petiole shows the typical concavo-convex outline in the transverse section and is surrounded on all sides by uniseriate glandular hairs (Fig. 3). The hairs are composed of a stalk of variable number of cells, usually 2–8, and a unicellular glandular head. The head is in most cases ellipsoidal or clavate, and in a few cases spherical (Figs. 10, *A*, *B*, *C*, *D*). The base of the hair is ordinarily formed of a single enlarged epidermal cell. The ellipsoidal head is the largest cell, measuring 55–135 microns in length which is sometimes greater than the length of the stalk. The hair thus has a total length of 120–175–225 microns.

The epidermis is covered by a thick cuticle which is continuous over the hairs as well. The epidermal cells are cubical with greater dimensions on the ventral side (30–56 microns by 22–45 microns) than on the dorsal one (24–40 microns by 20–34 microns). Following the epidermis is a thin layer of collenchyma of one to two layers of cells which are smaller on the dorsal side than on the ventral region. This is followed by a broad zone of parenchyma in which are distributed a number of vascular bundles. The parenchymatous cells are more or less round with distinct intercellular spaces and possess a diameter of 38–60–90 microns in transverse section.

The petiole receives three vascular bundles from the stem forming a dorsal arc in the middle. The two lateral bundles are larger than the central one. These bundles, however, split higher up into five and finally up to seven near the base of the leaf. In such cases, there are two-minute bundles, one on each side of the ventral groove while the remaining ones form a dorsal arc with the central bundle being the largest. Usually on the dorsal side of the bundles, there is some development of the mechanical tissue, which, however, is not distinct in case of the two lateral small bundles. Often the cells of the mechanical layer extend laterally to form a crescent ring outside the dorsal arc of the bundles.

Lamina.—Numerous multicellular hairs of the glandular type, as mentioned above, are present on both the surfaces of the leaf. The hairs with a spherical head and a stalk of four to five cells are more numerous, particularly within the interneural regions, while those with a clavate head and a stalk of 4–8 cells are more predominant on the veins.

The epidermis of the leaf is covered with rather a

thick cuticle which becomes continuous with that of the hair. The epidermal cells on the upper side are more or less cubical, measuring 16–32–40 microns by 24–38–56 microns in the transverse section, while those on the lower side are more or less tabular, being 24–45–56 microns by 24–36–50 microns. The outer walls of the epidermal cells show deposition of crystalline granules of calcium oxalate, beneath the cuticle. At places where this deposit is strongly developed, white patches on the surface of the leaf are formed. This deposition extends also to the walls of the hairs.

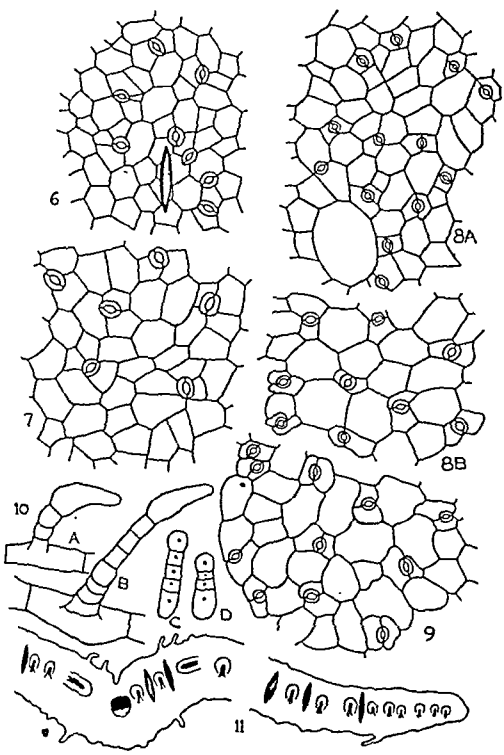

Fig. 6.—Upper epidermis of *Boerhaavia diffusa*, in surface view. ×80.

Fig. 7.—Lower epidermis of the same plant in surface view. ×80.

Fig. 8.—*A*, *B*. Upper epidermis of *Trianthema Portulacastrum* in surface view. ×80. Fig. 8 *A* shows smaller epidermal cells, in which are found bladder-like very big cells.

Fig. 9.—Lower epidermis of *Trianthema Portulacastrum* in surface view. ×80.

Fig. 10.—*A*, *B*, *C*, *D*. Four glandular hairs of *Boerhaavia diffusa*, the first two with a clavate-shaped head and the last two with a spherical head. ×110.

Fig. 11.—Transverse section of the leaf of *Boerhaavia diffusa*. ×20.

Stomata are present on both the surfaces of the leaf (Figs. 6 and 7). They show cuticular ridges but are free from the crystalline granules (Figs. 12 and 13). The distribution of stomata is greater on the upper surface than on the lower one (Table I). They are surrounded by 3–5, but more often by 4 ordinary epidermal cells, of which one is usually smaller than the rest. The stomata are small and measure 24–27–30 microns by $20^1/_2$–22–24 microns with their stomatal aperture $3^1/_2$–5 microns wide.

The epidermal cells in surface view, are all polygonal and straight-walled and measure 28–35–55 microns by 14–30–50 microns. The smaller epidermal cell, surrounding the stomata, however, is 18–22 microns by 9–14 microns.

TABLE I.—NUMBER OF STOMATA, HAIRS, AND EPIDERMAL CELLS IN *Boerhaavia diffusa* PER SQ. MM. LEAF SURFACE

Surface	Stomata	Hairs	Epidermal Cells
Upper	90–154–166	13–21–38	460–704–1014
Lower	66–110–126	11–22–30	302–496–662

The mesophyll is composed of a palisade layer on the upper side and a spongy tissue on the lower side of the leaf. The palisade cells are bent in such a way as to tend to converge round the bundle sheaths (Fig. 12), enclosing the veins. They are shorter in length about 50–56–70 microns, directly above the bundle sheath, but at its sides they become elongated and measure 70–85–90 microns by 12–20–24 microns. Those palisade cells which surround the bundle sheath on the dorsal or lower side of it, are the smallest and measure 32–40 microns by 14–22 microns. The spongy mesophyll consists of 2–4 layers of cells which are loosely arranged and are mostly oval or somewhat irregular, having a length of 30–56–78 microns and a breadth of 28–40–56 microns.

The lamina is traversed by numerous small veins, each surrounded by a bundle sheath which is, however, absent opposite to the phloem of the veins (Fig. 12). There are 4–5 cells of the bundle sheath, visible in the transverse section, mostly with a conical outline, their broad bases being away from the veins and measuring 25–32–40 microns directly above the veins and 45–56–66 microns toward the sides of the veins. However, those surrounding the veins which run longitudinally, appear palisade-like measuring 25–33 microns by 17–22 microns (Fig. 13). Bundles of long raphides traverse the mesophyll with their length lying in a dorso-ventral position (Fig. 11). They occur usually in between the veins and measure 140–170–190 microns in length.

The epidermal cells of the midrib are much smaller as compared to those of the lamina. As in the case of petiole, the epidermal cells are bigger on the ventral side and smaller on the dorsal surface. They measure 16–30–40 microns by 20–24–28 microns on the ventral side and 8–16–22 microns by 12–20–28 microns in the dorsal region. Following the epidermis of the midrib, on the dorsal side, is found a collenchyma of one to 2 layers of cells and a broad zone of parenchyma of 4–6 layers of cells. The cells of the collenchyma have only slightly greater dimensions than those of the epidermal cells,

but the parenchymatous cells are much bigger. The latter are mostly polyhedral or isodiametric with distinct intercellular spaces, measuring 22–38–45 microns in diameter.

A distinct mechanical tissue, 1–3 layers of cells thick, surrounds the vascular bundle of the midrib, dorsally. Near the base of the lamina, the midrib has 3–5 vascular bundles, but higher up it possesses only one bundle. These vascular bundles, however, are not surrounded by a distinct bundle sheath. The phloem of these bundles is composed of sieve tubes, companion cells, and phloem parenchyma, while the xylem consists of annular and spiral and sometimes of reticulate type of vessels and xylem parenchyma. On the ventral side of the vascular bundle, a small amount of parenchyma of 2 to 3 layers of cells or 2–3 palisade-like, but much wider cells, devoid of chloroplasts, are found, below the upper epidermis (Fig. 13).

Trianthema Portulacastrum

The morphological structures observed in both the red-flowered and white-flowered varieties are similar.

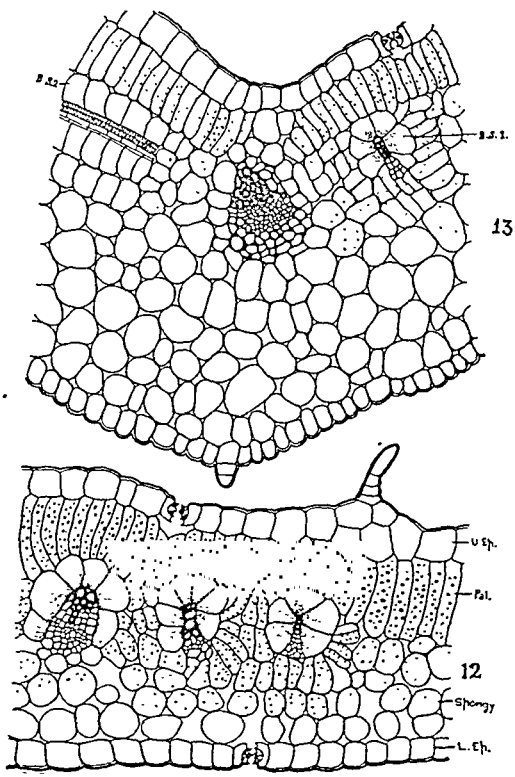

Fig. 12.—Details of the T. S. of lamina of *Boerhaavia diffusa*. ×80.

Fig. 13.—Details of the midrib of the same species. ×80.

U.Ep., upper epidermis; Pal., palisade layer; Spongy, spongy layer; L.Ep., lower epidermis; B.S.1, bundle sheath in transverse view; B.S.2, bundle sheath in longitudinal view.

Stem.—The transverse section of a young internode shows a somewhat plano-convex or slightly biconvex outline (Fig. 17). The single-layered epidermis is covered externally by rather a thin cuticle and numerous unicellular, but very much elongated hairs. These hairs are outgrowths of epidermal cells and measure 60–100–400 microns by 25–30–35 microns. Occasionally bicellular hairs are also found, in which case the second cell has a swollen bulb-like base, situated at the top of the first tapering cell. Rarely a hair may show in the middle region, transverse divisions, giving it a multicellular appearance. All these hairs are nonglandular and the bi- or multicellular ones attain a length up to 700 microns or even more.

Following the epidermis is a broad zone of cortex of 8–10 layers of cells which are parenchymatous with distinct intercellular spaces. In contrast to the epidermal cells which are small and cubical and measure 8–15–18 microns by 14–18–22 microns, the parenchymatous cells are very big and more or less isodiametric with a diameter of 22–50–80 microns. Some of these cells contain rosette crystals of calcium oxalate, 12–20 microns in diameter. A distinct endodermis is, however, not to be made out.

There are present a few layers of pericyclic cells on the outer side of the stele (Fig. 18). The stele is composed of a ring of vascular bundles 20 or more in number. Later when secondary growth takes place, a continuous cambium ring is formed. This cambium produces secondary xylem toward the inner side, and secondary phloem on the outer side opposite to the xylem. In the interfascicular region, however, conjunctive tissue or storage parenchyma is formed. The xylem vessels thus formed are of spiral, reticulate, and pitted type with simple pits on the walls. Sometimes a vessel with bordered pits may also be found. The secondary phloem consists of sieve tube tissue, companion cells, and phloem parenchyma. The conjunctive tissue formed during the secondary growth, in the interfascicular region, becomes thick walled and lignified. The outer cells of the pith lying in the vicinity of the vascular ring also become lignified and thus the stele presents, after secondary growth, a structure resembling that of the outer ring of vascular cylinder of *Boerhaavia diffusa*.

The cells of the pith are very large and parenchymatous with prominent intercellular spaces. They are isodiametric, measuring 70–120–160 microns in diameter. Some of these contain rosette crystals of calcium oxalate.

Petiole.—The outline of the petiole in the basal region of the leaf, is somewhat flat and less concavo-convex, due to the expansion of the sides of the petiole. In the upper region, approaching the lamina, it becomes more concavo-convex (Figs. 4 and 5). Externally it is covered by a very thin cuticle and surrounded by numerous unicellular and nonglandular hairs of the type mentioned above, measuring 60–120–225 microns in length. The epidermal cells on the ventral side are bigger (18–45–56 microns by 14–24–30 microns) than those on the

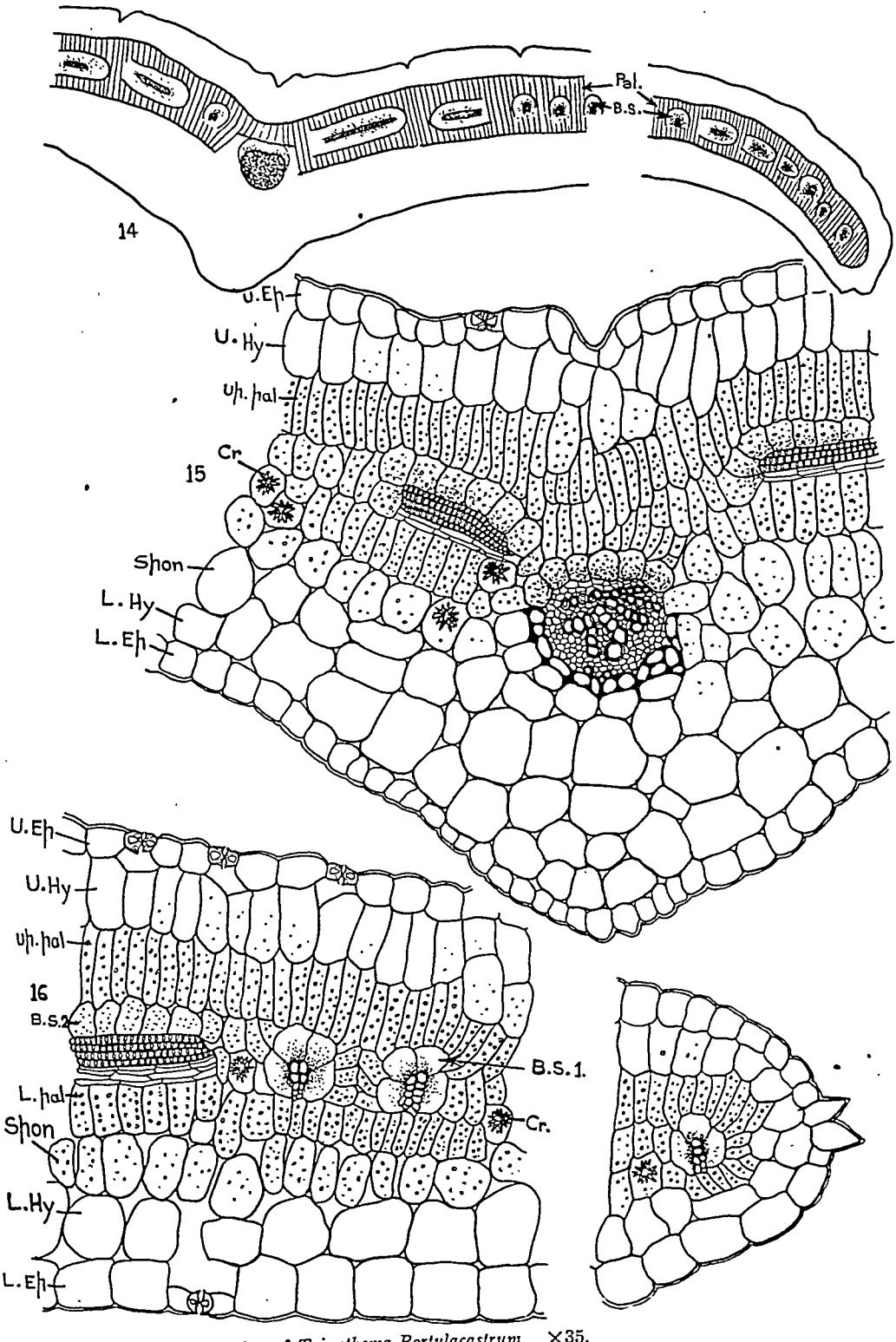

Fig. 14.—Transverse section of *Trianthema Portulacastrum*. ×35.
Fig. 15.—Details of the T. S. of the lamina of the same species. ×150.
Fig. 16.—Details of the T. S. of the midrib of the same species. ×150.
U.Ep., upper epidermis; U.Hy., upper hypodermis; Up.pal., upper palisade layer; L.Hy., lower hypodermis; L.Ep., lower epidermis; B.S.1, bundle sheath in transverse view; B.S.2, bundle sheath in longitudinal view; Cr., rosette crystal of calcium oxalate.

directly above the veins and 48–60 microns toward the sides of the veins.

The midrib of *Trianthema Portulacastrum*, like that of *Boerhaavia diffusa*, has bigger epidermal cells on the ventral side (20–30–42 microns by 28–34–50 microns) as compared to those on the dorsal surface (17–25–35 microns by 14–26–36 microns). Collenchyma is, however, absent on the dorsal side. The parenchymatous cells on the dorsal side are 3–5 layers thick, polyhedral, and measure 30–50–68 microns by 25–34–57 microns. The mechanical tissue, surrounding the vein dorsally, is only one or at places two layers of cells thick. On the ventral side of the midrib, the cells are found arranged more or less in the same order as in the lamina of the leaf, that is, a single layer of hypodermis below the upper epidermis, two layers of palisade cells, and then the bundle sheath enclosing the xylem of the veins.

The vascular bundle of the midrib consists, as usual, of xylem on the ventral side and phloem on the dorsal side. In the lower parts of the leaf, particularly near the base, the vascular bundle breaks up into three or four small bundles.

Near the margin of the leaf, the arrangement of the cells are the same as in the lamina, but the epidermal cells of the margin are often enlarged to form a cone-shaped cell structure (Fig. 16). In the red variety of Trianthema, the epidermal cells of the margin contain a pinkish coloring matter which gives the margin of the leaf a pinkish tinge, characteristic of this variety.

In order to bring out further the diagnostic features of these two species, their stomatal indices and palisade ratios were determined. A glance at Table III, which shows the stomatal indices obtained in both the cases, clearly indicates that *Trianthema Portulacastrum* has much higher values than *Boerhaavia diffusa*.

It will be seen from the analysis of variance in Table III that the variation due to species is highly significant. No significant variance exists either between the upper and the lower surfaces, or between the localities. The interaction between species and surface is also not significant. Thus the two species can be differentiated from each other by their stomatal indices, as also by their palisade ratios given in Table IV.

From the data given above, though it is obvious that *T. Portulacastrum* has significantly higher palisade ratios than *B. diffusa*, yet statistical analysis was made primarily to determine if there were significant differences between localities and whether the interaction between locality and species was significant. The analysis of variance in Table IV clearly shows that the effect of locality or the interaction between locality and species is not significant.

TABLE III.—STOMATAL INDICES OF *Boerhaavia diffusa* AND *Trianthema Portulacastrum* COLLECTED FROM DIFFERENT LOCALITIES

I		II		III		IV[a]	
Upper Surface	Lower Surface	Upper Surface	Lower Surface	Upper Surface	Lower Surface	Upper Surface	Lower Surface
			Boerhaavia diffusa				
15.0	14.3	15.1	14.3	15.2	12.7	14.8	12.6
11.7	12.0	14.3	11.3	16.5	17.1	13.6	11.8
12.8	11.2	15.8	18.3	14.3	16.5	15.6	14.6
12.0	13.0	15.2	13.2	12.0	15.8	12.8	15.3
12.9	11.1	16.6	17.5	12.9	13.4	11.9	14.8
			Trianthema Portulacastrum				
16.2	17.1	17.5	14.6	20.4	18.6	17.8	16.8
16.4	15.9	19.6	19.3	15.8	19.5	19.5	18.5
14.6	18.7	15.6	15.7	21.1	20.6	18.3	16.0
15.8	16.5	22.4	18.3	19.8	18.8	17.2	17.3
17.6	15.6	21.7	19.4	17.6	19.5	16.5	16.8

Analysis of Variance

Variation Due to	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Locality	3	75.5210	25.174
Species	1	292.2300	292.230
Surface	1	0.7772	0.7772
Species × surface	1	0.7833	0.7833
Error 1	9	144.5960	16.0662
Error 2	64	62.2025	0.9719
Total	79	576.1100	

[a] Figures I, II, III, and IV indicate four localities.

DISCUSSION

From what has been mentioned above, it will be evident that the two species, namely, *Boerhaava diffusa* and *Trianthema Portulacastrum*, resemble

TABLE IV.—PALISADE RATIOS OF *Boerhaavia diffusa* AND *Trianthema Portulacastrum* COLLECTED FROM DIFFERENT LOCALITIES

	Boerhaavia diffusa Locality				*Trianthema Portulacastrum* Locality			
	I	II	III	IV	I	II	III	IV
	6.00	6.50	6.00	6.25	12.00	13.00	12.50	12.00
	4.00	3.50	4.25	3.50	12.50	11.75	13.00	12.75
	4.50	3.25	5.00	4.75	11.25	12.50	13.50	11.50
	3.50	4.50	3.50	4.50	10.25	10.50	12.00	10.00
	4.50	4.00	4.50	5.00	10.50	11.00	13.00	12.00
Mean	4.50	4.35	4.65	4.80	11.30	11.75	12.80	11.65

Analysis of Variance

Variation Due to	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Locality	3	3.6375	1.2125
Species	1	532.675	532.675
Locality × species	3	3.1875	1.0625
Error	32	31.045	0.9702
Total	39	570.545	

each other in their anatomical features. But for the anatomy of the stem which differs widely in the two cases, inasmuch as there is no anomalous growth in the stem of *T. Portulacastrum* the leaf presents very close similarity in both the species. The occurrence of bundle sheath and the arrangement of palisade layers around it are more or less similar. Even the number of vascular bundles in the petiole of *Boerhaavia diffusa* particularly near the base of the lamina, where the 3 vascular bundles split into 5, is the same as observed in case of *Trianthema Portulacastrum*at similar positions. Despite such close resemblance, there are certain important differences by which the stem and the leaf of these species could be easily distinguished, as shown below:

Boerhaavia diffusa	*Trianthema Portulacastrum*
Stem Structure	
1. There is an outer covering of multicellular, glandular hairs, composed of a stalk of 6–12 cells and a clavate-shaped head.	The hairy covering consists of nonglandular and unicellular hairs of great length. Rarely bi- or multicellular hairs are found.
2. The epidermal cells are fairly big, so that the ratio of these cells to the parenchymatous cortical cells is less.	The epidermal cells are smaller and the parenchymatous cells are much bigger. Hence the difference between the size of these two types of cells is striking.
3. There is one to two layers of collenchyma below the epidermis.	Collenchyma is absent.
4. The stele, in the young stem, consists of two large central bundles, surrounded by a ring of 6–14 loosely arranged bundles and an outer ring of very small bundles 15–20 or more in number. During secondary growth, the outer ring becomes continuous and later, additional cambium rings arise which produce four or five more growth rings.	The stele, in the young stem, consists of a ring of 20 or more small bundles which during secondary growth become continuous.
Leaf Structure	
5. The shape of the petiole is of the normal type, being concavo-convex throughout, in the transverse section.	The cross section of the petiole is flattened at the basal region of the leaf, but near the lamina it becomes concavo-convex.
6. Glandular hairs of the type as found on the stem, but with a shorter stalk, occur on the petiole. The lamina, in the interneural regions, possesses typical glandular hairs 3–4 celled with a spherical head, but on the veins such hairs as have a clavate head are found.	Similar hairs as are found on the stems are present on the petiole as well as the lamina of the leaf.
7. The petiole is supplied with three vascular bundles which split into five and then into seven, near the base of the lamina.	There are five vascular bundles throughout the petiole.
8. Stomata, surrounded by 3–5 ordinary epidermal cells, are found on both the surfaces. Both the stomata and epidermal cells are smaller in size, but greater in number per sq. mm. leaf area, the upper surface having 154 stomata and 703 epidermal cells (av.); the lower, 110 stomata and 496 epidermal cells.	Stomata, surrounded by 2 subsidiary cells, are present on both the surfaces. The stomata and epidermal cells are bigger and less in number per sq. mm. leaf area, the upper surface having 65 stomata and 350 epidermal cells at an average, while the lower one has 40 stomata and 205 epidermal cells.

9. Hypodermis is absent.	A distinct hypodermis is present below each epidermis.
10. The spongy mesophyll is 2–3 layers thick and loosely arranged.	The spongy mesophyll consists of 1–2 layers of cells which are more compactly arranged.
11. Bundles of raphides traverse the whole mesophyll dorso-ventrally.	Rosette crystals are found both in the stem and the leaf.
12. The midrib shows 3–5 vascular bundles in the lower part of the leaf, but higher up there is only one bundle, and the bundle sheath is absent.	There is only one vascular bundle throughout the midrib and it is surrounded by bundle sheath.
13. The ventral side of the midrib possesses parenchymatous cells.	The ventral side of the midrib has palisade cells like the lamina of the leaf.
14. The range of stomatal indices and palisade ratios is lower.	This range is higher in *Trianthema Portulacastrum*, as shown in Table V.

TABLE V.—RANGE OF VARIATION IN THE STOMATAL INDICES AND PALISADE RATIOS OF *Boerhaavia diffusa* AND *Trianthema Portulacastrum*

Species	Stomatal Indices: Upper Surface	Stomatal Indices: Lower Surface	Palisade Ratios
Boerhaavia diffusa	11.7–13.2–16.0	11.2–14.3–18.3	3.25–4.50–6.25
Trianthema Portulacastrum	14.6–17.2–22.4	15.6–18.3–20.6	10.25–12.00–13.00

SUMMARY

The macro- and microscopical features of the stem and the leaf of *Boerhaavia diffusa* and *Trianthema Portulacastrum* have been studied, with a view to bringing out the distinguishing pharmacognostical features of these species.

The young stem of *Boerhaavia diffusa* is covered externally by characteristic glandular hairs and consists of a layer of epidermis, 1–2 layers of collenchyma, and a broad zone of parenchyma in which vascular bundles are distributed like a monocot stem. There is anomalous wood formed by the renewed activity of the parenchyma formed by the first cambium.

The petiole also is surrounded by similar glandular hairs and consists of 1–2 layers of collenchyma and a broad zone of parenchyma in which are found 3–7 vascular bundles and long raphides of calcium oxalate. The leaf is typically dorsi-ventral with 1 to 2 layers of palisade cells on the ventral side and 3–4 layers of spongy cells on the dorsal side. The vascular bundles are surrounded by bundle-sheaths round which the palisade cells tend to converge. The bundle-sheath is absent opposite to the phloem of the veins. The lower part of the leaf has 3–5 vascular bundles in the midrib, but higher up there is only one bundle. Long bundles of raphides traverse the mesophyll of the leaf.

The young stem of *Trianthema Portulacastrum* is surrounded externally by long, unicellular, and nonglandular hairs which are present also on the petiole and the lamina of the leaf. Collenchyma is absent in the stem, but there are well-developed parenchymatous cells, some of which contain rosette crystals of calcium oxalate. Distributed in the parenchyma is a ring of 20 or more vascular bundles which after secondary growth, become continuous.

The petiole is very much flattened at its base, due to lateral expansions, and contains rosette crystals. There are 5 vascular bundles distributed in the parenchyma of the petiole. The leaf of *Trianthema Portulacastrum* resembles anatomically that of *Boerhaavia diffusa*. It is of dorsi-ventral type with palisade and spongy cells arranged as in *B. diffusa*. The spongy mesophyll is, however, more compact and represented by 1–2 layers of cells which contain also rosette crystals. The veins of the leaf are all surrounded by bundle sheaths which are absent in the phloem portions of the veins.

The two species differ widely in their stomatal indices and palisade ratios, *Trianthema Portulacastrum*, possessing higher values in these regards. Other important differences between the stems and leaves of these two species have been discussed.

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The Estimation of Penicillin K in Commercial Penicillin*

By WILLIAM W. WRIGHT and DONALD C. GROVE

A method for the estimation of penicillin K in commercial penicillin together with its development, calculations, and results obtained on commercial samples is presented.

THE PURPOSE of this paper is to present the experimental data which led to the development of the method now being used by the U. S. Food and Drug Administration for the determination of penicillin K, the calculations involved and some results obtained by the method on commercial penicillin.

METHOD

Dilute a weighed sample or the contents of a vial of penicillin with 0.3 *M* phosphate (Na_2HPO_4 and KH_2PO_4) buffer *p*H 6.0, to give a solution containing approximately 0.6 mg. (1000 units) per ml. In the case of calcium penicillin where a precipitate of calcium phosphate occurs, remove the precipitate by filtration and use the clear filtrate. Place a 15.0-ml. aliquot of this solution in a 125-ml. separatory funnel, add 30.0 ml. of chloroform U. S. P., and shake for one minute. (Carry out all operations at room temperature.) Allow the mixture to stand, with occasional swirling to settle the droplets of chloroform, until the top layer is clear (usually about ten minutes). Draw off all but about 2 ml. of the lower chloroform layer through a small pledget of cotton into a glass-stoppered flask. Take two 4.0-ml. aliquots of the original solution, two 4.0-ml. aliquots of the buffer solution remaining in the separatory funnel, and two 10.0-ml. aliquots of the chloroform solution. Each aliquot is placed in a 125-ml. glass-stoppered Erlenmeyer flask. To one of the two aliquots of each solution add 4.0 ml. of 1 *N* NaOH and allow to stand at room temperature for fifteen minutes. At the end of this time, add 4.0 ml 1.2 *N* HCl and 10.0 ml. of 0.01 *N* I_2 (prepared from 0.1 *N* I_2 U. S. P.). After fifteen minutes titrate the excess iodine using 0.01 *N* $Na_2S_2O_3$ (prepared from 0.1 *N* $Na_2S_2O_3$ U. S. P.). To each of the other aliquots add 10.0 ml. 0.01 *N* I_2 and titrate immediately with 0.01 *N* NaS_2O_3 for the blank determinations. Regard the difference in titers divided by 2.52 as the milligrams of penicillin sodium salt. Calculate the per cent penicillin in the buffer layer and in the chloroform layer as compared to the original solution. The sum of these percentages should be 100% ± 2%. The per cent penicillin K = (96.92 + % in chloroform − % in buffer) × 1.67 (formula A).

The method was developed from observations made by Wattel (1), that at certain hydrogen-ion concentrations penicillin K was extracted by immiscible solvents to a greater extent than the other types of penicillin. Preliminary observations in our laboratories showed that when a 0.3 *M* phosphate buffer solution of crystalline penicillin K[1] at *p*H 5.0 was shaken with two volumes of chloroform, the penicillin K distributed itself so that about 75% was found in the chloroform while about 25% remained in the aqueous phase. Crystalline penicillin G (FDA working standard) under the same conditions

* Received Nov. 1, 1947, from the Division of Penicillin C[illegible] Administration, F[illegible] A part of a dis- se[illegible] r of Philosophy d[illegible] D. C.

[1] Manufactured by Charles Pfizer and Company, Inc., and obtained through the courtesy of the Antibiotics Study Section, U. S. Public Health Service.

distributed itself so that about 14% was found in the chloroform and about 86% remained in the aqueous phase. Experiments at higher hydrogen-ion concentrations were carried out to determine whether a point could be reached where a fairly high percentage of penicillin K could be extracted with little or no penicillin G: at *p*H 5.5 the chloroform extracted about 60% penicillin K and 5% penicillin G, at *p*H 6.0 about 31% K and 1.5% G, and at *p*H 6.5 about 11% K and 0.7% G. Extraction at *p*H 6.0 was chosen because at *p*H 5.5 too much G was extracted and at *p*H 6.5 too little K was extracted. The iodometric assay method of Alicino (2) as modified by Mundell, *et al.* (3) for determining the potency of the penicillin was chosen because the chloroform extract could be assayed directly without an additional shakeout into buffer as is required when the bioassay method is used.

Table I shows the results obtained by the above method using penicillin K alone and penicillin G alone.

TABLE I.—DISTRIBUTION RESULTS OBTAINED AT *p*H 6.0 ON PENICILLIN K AND G

Penicillin	% in Buffer Layer	% in $CHCl_3$	% Recovery
K	68.36	28.22	96.58
K	68.76	32.10	100.86
K	67.92	32 77	100 69
K	67.99	31.93	99.92
K	69.77	32.48	102.25
Av. of 5	68.56	31.50	100.06
G	98.06	1.14	99.20
G	97.40	1.53	98.93
G	97.80	1.45	99.25
G	96 33	1.95	98.28
Av. of 4	97.40	1.52	98.92

It will be noted that the average recovery in both layers in the case of penicillin K was 100.06% and in the case of penicillin G 98.2%. Correcting the figures on the basis of 100% recovery, the following distribution figures are obtained.

Penicillin	% of Total in Aqueous Layer	% of Total in $CHCl_3$
K	68.52	31.48
G	98.46	1.54

Having established the distribution figures for penicillin K and G, the percentage of penicillin K in an unknown sample of penicillin can be calculated on a weight basis from either of the following formulas.

$$\% K = \frac{(98.46 - \% \text{ in aqueous})}{(98.46 - 68.52)} \times 100 \text{ or } (98.46 - \% \text{ in aqueous})\ 3.340 \quad (B)$$

$$\% K = \frac{(\% \text{ in chloroform} - 1.54)}{(31.48 - 1.54)} \times 100 \text{ or } (\% \text{ in chloroform} - 1.54)\ 3.340 \quad (C)$$

In actual practice the sum of the amount in the chloroform layer and the amount in the buffer layer is not always exactly 100%, therefore there will be some variation in the per cent of penicillin K depending upon whether one calculates this percentage from the amount remaining in the aqueous buffer solution or the amount extracted by the chloroform using the above two formulas. For this reason an average of the sum of the per cent K obtained by both of the above formulas is used to obtain the per cent K, as expressed by the following:

$$\% K = (96.92 + \% \text{ in chloroform} - \% \text{ in buffer}) \times 1.67 \quad (A)$$

Separate solutions of penicillin G and penicillin K were prepared in 0.3 *M* phosphate buffer *p*H 6.0 to give a concentration of approximately 0.6 mg. per ml. Each solution was assayed for milligrams per milliliter by the iodometric method and then the two solutions were mixed in varying proportions and the mixtures assayed for the per cent of penicillin K by the above method. The results obtained are shown on Table II.

TABLE II.—RESULTS OBTAINED ON MIXTURES OF PENICILLIN K AND G

Known K and G in Mixture, %		Penicillin Found in Each Layer, %		Penicillin K Found Using Formula A, %
K	G	In Buffer	In $CHCl_3$	
0	100	98.46	1.54	0.0
10.2	89.8	95.49	4.51	9.9
21.4	78.6	93.68	7.20	17.4
36.2	63.8	86.76	11.72	36.5
49.8	50.2	83.39	16.61	50.3
100	0	68.52	31.48	100.0

It was desirable next to determine what effect the other penicillin —X, F, and dihydro F—would have on the method for the determination of penicillin K. Accordingly, these different types of penicillin were each run through the procedure separately and the per cent in the buffer and in the chloroform determined. Table III gives a summary of the amount found in each layer for each type penicillin. It will be noted that penicillin X will not interfere. Penicillins F and dihydro F are somewhat more soluble in chloroform than penicillin G; however, the error introduced by the presence of these two types of penicillin is very small.

TABLE III.—DISTRIBUTION OF VARIOUS PENICILLINS BETWEEN CHLOROFORM AND 0.3 *M* *p*H 6 0 BUFFER

Penicillin	Penicillin Found in Each Layer, %		Using Formula A, % K Apparently Present
	In Buffer	In $CHCl_3$	
G	98.46	1.54	0.0
X[a]	98.52	1.00	1.0
F[a]	96.28	2.16	4.7
Dihydro F[a]	96.18	2.33	5.2
K	68.52	31.48	100.0

[a] The penicillin F was manufactured by the Upjohn Company and the penicillin dihydro F by Chas. Pfizer and Company, Inc. Both samples were obtained through the courtesy of the Antibiotics Study Section, U. S. Public Health Service. The penicillin X was obtained through the courtesy of Lederle Laboratories.

Tables IV and V give some typical penicillin K results obtained on commercial lots of sodium and calcium penicillin, respectively. It may be said that the penicillin K content of penicillin being distributed at the present time is very small.

TABLE IV.—SODIUM PENICILLIN

Manufacturer	Lot No.	Date Tested	% K
A	703A007	5-1-47	0.0
	704A001	5-12-47	2.5
	704A002	5-26-47	1.9
	705A003	6-9-47	3.2
	705A004	6-11-47	3.7
B	5479	5-22-47	2.8
	54715	5-22-47	0.0
	54719	5-30-47	0.2
	54726	6-5-47	1.4
	6475	6-13-47	0.0
C	C7690	5-8-47	0.0
	E7688	5-23-47	1.6
	E7687	5-23-47	2.2
	E7690	5-26-47	3.2
	E7616	6-11-47	5.1
D	1502	7-4-47	0.0
	1513	7-4-47	6.5
	1514	7-4-47	7.9
	1517	7-4-47	6.7
	1518	7-4-47	5.3
E	3810-837A	6-25-47	5.3
	3810-891A	6-30-47	7.5
	7810-881C	7-1-47	10.4
	7810-881D	7-2-47	12.1
	7810-877D	7-3-47	5.1
		Av.	3.8

TABLE V.—CALCIUM PENICILLIN

Manufacturer	Lot No.	Date Tested	% K
A	705X902	6-11-47	2.1
	706X900	6-17-47	0.0
	706A001	6-23-47	1.2
	706X901	6-25-47	2.2
	706X902	7-4-47	0.0
B	1591	5-26-47	5.7
	1701	6-11-47	5.5
	1702	6-11-47	1.8
	1703	6-14-47	1.4
	1704	6-14-47	4.3
C	1117C	5-3-47	0.0
	1427C	6-19-47	2.1
	1457C	6-23-47	2.2
	1477C	6-25-47	0.8
	1517C	7-1-47	5.2
D	1505	6-5-47	1.7
	1527	6-24-47	2.3
	1528	6-24-47	4.4
	1533	6-25-47	5.6
	1534	6-25-47	0.3
E	2S245	6-10-47	1.7
	18S177	6-10-47	4.8
	18S178	6-11-47	0.0
	2S247	6-19-47	7.1
	2S248	7-5-47	5.0
F	471952	6-24-47	11.4
	472916	6-25-47	6.8
	472917	7-2-47	14.1
	472918	7-4-47	5.5
	471957	7-4-47	18.8
G	CO619	6-17-47	0.0
	CO677	6-19-47	0.0
	CO686	6-25-47	0.0
	CO749	6-27-47	2.3
	CO759	7-3-47	0 7
		Av.	3 6

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

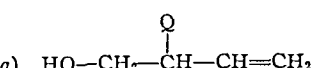
Montanic acid
Glucoascorbic acid
Acenaphthylene
Cuscohygrine
Hemipinic acid
Hordenine
4-Hydroxyquinoline-3-carboxylic acid
4-Phenyl-2-aminobutyric acid
Undecyl aldehyde
Scopoletin
N-Methylnipecotic acid
Ethyl vanillate
Thiocytosine
Phosphatase, purified
Hydroxylamine-O-acetic acid hydrochloride
Methoxinine
d-Erythrose
d-Threose
17-Hydroxycorticosterone
11-Dehydro-17-hydroxycorticosterone
Stigmasterol
Maltobionic acid

The Preparation of Certain Substituted Alkenols and Their Chloro Analogs*

By FRED SEMENIUK† and GLENN L. JENKINS

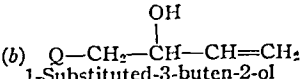
CERTAIN olefinic halogenated compounds are of significance from the standpoint of preparative methods proposed for them and of their possible utility as intermediates in the synthesis of new types of physiologically active compounds. These halogenated compounds are the 4-substituted-1-halo-2-butenes, which are satisfied by the general formula: Q—CH_2—CH=CH—CH_2—X, where Q— is any substituent not necessarily hydrocarbonaceous. Those selected for preliminary study constitute a fairly uniform series of five in which the various values of Q— are methyl-, cyclohexyl-, phenyl-, α-naphthyl- and *o*-ethoxyphenyl-, and in which the halo-substituent is chloro-.

3,4-Epoxy-1-butene (Butadiene monoxide), H_2C — O — CH — CH=CH_2, is a compound of fair availability in recent years. Its reactive and especially additive properties are many and variable. Among many others, it will add Grignard reagents—a characteristic of epoxy compounds; the manner in which this addition takes place with respect to the direction of opening of the epoxy ring is debatable, so that on hydrolysis subsequent to addition, the formation of either or both of two possible alcohols must be considered, corresponding to the structures:



(*a*) HO—CH_2—CH(Q)—CH=CH_2
2-Substituted-3-butenol

and



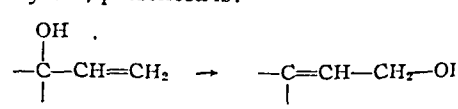
(*b*) Q—CH_2—CH(OH)—CH=CH_2
1-Substituted-3-buten-2-ol
(Vinyl-substituted-methyl-carbinol)

where Q—Mg—X is the Grignard reagent employed.

Using methylmagnesium iodide as the Grignard reagent, adding to 3,4-epoxy-1-butene, and following the addition with hydrolysis (acid catalyzed), we obtained a pentenol whose constants (b. p. 133–139°/747 mm.; m. p. of 3,5-dinitrobenzoate: 59–60°) corresponded to neither 2-methyl-3-butenol (isomeric form *a*), (b. p. 120–121°/756 mm.; m. p. of 3,5-dinitrobenzoate: 57.5–58°) (1), nor 1-methyl-3-buten-2-ol (vinylethylcarbinol) (isomeric form *b*), (b. p. 111–118°/ordinary, or no pressure specified) (2–6). Our pentenol, however, did correspond to the constants (b. p. 138–142°/no pressure specified) (5, 7–9) for a structure of the isomeric form: H_3C—CH_2—CH=CH—CH_2—OH (4-methyl-2-butenol, 2-pentenol, β-ethylallyl alcohol).

A reasonable explanation for the formation of 4-methyl-2-butenol, but not of 2-methyl-3-butenol (which could not be isolated), is that a combination of two events is involved: (*a*) the epoxy ring of 3,4-epoxy-1-butene opens to Grignard reagents to form, preferentially, secondary alcohols when the addition product is hydrolyzed; (*b*) if the hydrolysis is acid catalyzed, the secondary alcohols undergo allylic rearrangement to β-substituted-allyl alcohols which correspond to the 4-substituted-2-butenols; these in turn are obvious precursors for the preparation of the 4-substituted-1-halo-2-butenes.

Allylic rearrangements have been experimentally established for a variety of susceptible classes of compounds, among which are the vinylalkylcarbinols, vinylarylcarbinols, and vinyldialkylcarbinols. The general equation by which the rearrangement may be represented is:



Delaby (10) and Prevost (5, 8, 11) have described its application to vinylalkylcarbinols. An extension of the rearrangement

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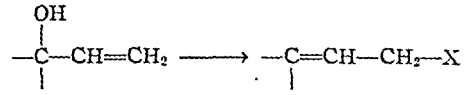
is applicable in terms of the more general equation:



representing the preparation of rearranged analogs. The effective agents of conversion and of rearrangement of this type are HX, PCl_3, PBr_3, and $SOCl_2$ (12–14).

The 1,4-addition of Grignard reagents to 3,4-epoxy-1-butene cannot be denied as a possible reaction mechanism to explain the formation of 4-substituted-2-butenols without relying entirely upon the incidence of allylic rearrangement. Furthermore, it serves to explain the fact that alcohols of but one isomeric form could be isolated.

3,4-Epoxy-1-butene proved to be an interesting intermediate in the route of synthesis employed. Certain difficulties were encountered in the manipulative procedure, the most notable of which were the extreme exothermy, high viscosity and foaming encountered in the reaction mixtures during the addition of the 3,4-epoxy-1-butene to the prepared Grignard reagents. It was found more desirable to employ less cooling of the reaction flask at the expense of time required for adding the 3,4-epoxy-1-butene to the Grignard reagent, in order to obviate the viscosities which made stirring both difficult and dangerous from the standpoint of sudden foaming which resulted and of the incidence of violent local action due to inadequate stirring.

The five alcohols prepared have been assigned the same general formula, corresponding to the isomeric form represented by the name 4-substituted-2-butenol, for the reason that the same general procedure was used in the preparation of all of them. However, minor variations in the procedure were necessary for certain individual members.

Of the five alcohols, the analogs of radical weight greater than methyl- are new. They were derivatized as their 3,5-dinitrobenzoates; the derivatives were analyzed for % N, the results checking satisfactorily with calculated values.

For the conversion of the five 4-substituted-2-butenols to their corresponding chloro analogs, the 4-substituted-1-chloro-2-butenes, thionyl chloride was used as the conversion agent. No rearrangements were anticipated since thionyl chloride favors the same isomeric form (14).

Of the five 4-substituted-1-chloro-2-butenes prepared, the analogs of radical weight greater than methyl- are new. The series showed a notable tendency to decompose with the liberation of HCl. This was demonstrated by their precipitation of AgCl immediately in the cold with aqueous or alcoholic silver nitrate solution, by formation of dimethylammonium chloride exclusively, on reaction with dimethylamine, and by consistently low (1–4 percentage points) analyses for % Cl. Attempts to prepare Grignard reagents of them for derivation of their anilides intended for analyses for % N to complement the % Cl analyses, failed, even under the strenuous conditions recommended by Underwood and Gale (15).

Inasmuch as R—Br compounds generally form Grignard reagents more readily than do the corresponding R—Cl compounds (16, 17), and as 3,4-epoxy-1-butene has proved to be a satisfactory intermediate in the preparation of this class of compounds, it is proposed to prepare the bromo-analogs of the 4-substituted-1-chloro-2-butenes.

EXPERIMENTAL

In the preparation of the 4-substituted-2-butenols ground glass equipment and a motor-driven mercury-seal stirrer were used.

1. **4-Methyl-2-butenol (2-Pentenol, β-Ethylallyl alcohol) (I).**—To 300 cc. anhydrous ether containing 48.6 Gm. (2 atoms) magnesium turnings and a crystal of iodine was added 284 Gm. (2 moles) methyl iodide at a rate governing smooth Grignard reagent formation. Mild heat and stirring were applied for 8 hours after spontaneous reaction. While keeping the reaction flask efficiently cooled in an ice-salt bath, 140 Gm. (2 moles) 3,4-epoxy-1-butene was added dropwise with stirring. Violent reaction, liberation of heat and dense white fumes, and extreme foaming occurred. These were governed by interruptions in additions and stirring. The total addition took eight hours. The bath was removed and stirring was continued for six hours. The reaction mixture was poured onto 1 Kg. of cracked ice and hydrolyzed by stirring into it 1 L. diluted H_2SO_4. The ether layer was salted out, separated, and evaporated free of ether. The resi-

due was dried with anhydrous $CaCl_2$, separated, and distilled. The fraction boiling steadily distilled at 133–139°/747 mm., 50–55°/12 mm. A pale yellow liquid with a pungent, garlic odor.

Yield: 61.4 Gm. (35.7%); d_4^{20} = 0.859; n_D^{25} = 1.4325. M. p. of 3,5 dinitrobenzoate 59–60°.

Anal.—Calcd. for 3,5-dinitrobenzoate ($C_{12}H_{12}O_6N_2$): N, 10.00. Found: N, 10.45, 10.54.

2. **4-Cyclohexyl-2-butenol (II).**—Using the same general procedure as for the preparation of (I), 165.5 Gm. (1.4 moles) chlorocyclohexane, 34.0 Gm. (1.4 atoms) magnesium turnings and 98 Gm. (1.4 moles) 3,4-epoxy-1-butene yielded, when the dried residue from the evaporation of the ether was submitted to purification by vacuum distillation (glass wool was used in the distillation flask to prevent violent bumping and obtain steady distillation) the fraction boiling steadily at 115–120°/13 mm. A pale yellow liquid.

Yield: 73.3 Gm. (34%); d_4^{20} = 0.939; n_D^{25} = 1.4795. M. p. of 3,5 dinitrobenzoate 106–107°.

Anal.—Calcd. for 3,5-dinitrobenzoate ($C_{17}H_{20}O_6N_2$): N, 8.05. Found: N, 8.09, 8.16.

3. **4-Phenyl-2-butenol (III).**—Using the same general procedure as for the preparation of (I), 314 Gm. (2 moles) bromobenzene, 48.6 Gm. (2 atoms) magnesium turnings and 140 Gm. (2 moles) 3,4-epoxy-1-butene yielded, when the dried residue from the evaporation of the ether was submitted to purification by vacuum distillation, the fraction boiling steadily at 120–125°/12 mm. The product is a pale yellow liquid.

Yield: 112.7 Gm. (38%); d_4^{20} = 1.006; n_D^{25} = 1.5390. M. p. of 3,5 dinitrobenzoate 104–105°.

Anal.—Calcd. for 3,5-dinitrobenzoate ($C_{17}H_{14}O_6N_2$): N, 8.19. Found: N, 8.66, 8.47.

4. **4-α-Naphthyl-2-butenol (IV).**—To 300 cc. anhydrous ether containing 41 Gm. (1.68 atoms) magnesium turnings and a crystal of iodine was added 349 Gm. (1.68 moles) α-bromonaphthalene at such a rate as to keep Grignard reagent formation progressing. Following spontaneous reaction, which was incomplete, as strong heat as was safe, and moderate stirring were applied for twenty-four hours. The reaction flask was immersed in an ice bath and 117.6 Gm. (1.68 moles) 3,4-epoxy-1-butene was added dropwise with stirring. The reaction was violent (dense white fumes and heat, but no foaming) and was governed by interruptions in addition and stirring. The total addition took twelve hours. The ice bath was removed and stirring was continued for twelve hours. The reaction mixture was poured onto 1 Kg. of cracked ice and hydrolyzed with 1 L. diluted H_2SO_4. The ether layer was salted out, separated, and evaporated free of ether. The residue was dried with anhydrous $CaCl_2$, separated, and distilled. The fraction boiling steadily distilled at 163–168°/3 mm. A viscous, pale yellow liquid.

Yield: 100.5 Gm. (30.2%); d_4^{20} = 1.114; n_D^{25} = 1.6168. M. p. of 3,5 dinitrobenzoate 179–180°.

Anal.—Calcd. for 3,5-dinitrobenzoate ($C_{21}H_{16}O_6N_2$): N, 7.15. Found: N, 7.57, 7.73.

5. **4-o-Ethoxyphenyl-2-butenol (V).**—To 300 cc. anhydrous ether containing 48.6 Gm. (2 atoms) magnesium turnings and a crystal of iodine was added 313.2 Gm. (2 moles) o-chlorophenetole at such a rate as to keep Grignard reagent formation progressing. Great difficulty was encountered in attempting to get the Grignard reagent to form; the addition of methyl iodide and of more iodine, and finally the application of strong heat and constant stirring for forty-eight hours had to be resorted to. At the end of this time almost all the magnesium had reacted. The reaction flask was cooled and 140 Gm. (2 moles) 3,4-epoxy-1-butene was added slowly, dropwise with stirring. The amount of cooling was only slight, since excessive cooling made the reaction mixture too viscous for stirring. Total addition took eight hours. Stirring was continued six hours longer with the application of mild heat to maintain the fluidity of the reaction mixture. The reaction mixture was hydrolyzed in the usual way, using 1 Kg. cracked ice and 1 L. diluted H_2SO_4. This was followed by salting out, separation, evaporation of the ether, drying of the residue, separation, and finally purification by vacuum distillation. The fraction boiling steadily distilled at 132–137°/3 mm. The product is a viscous, pale yellow liquid.

Yield: 56.5 Gm. (14.7%); d_4^{20} = 1.040; n_D^{25} = 1.5377. M. p. of 3,5 dinitrobenzoate 79°.

Anal.—Calcd. for 3,5-dinitrobenzoate ($C_{19}H_{18}O_7N_2$): N, 7.26. Found: N, 7.99, 7.72.

6. **4-Methyl-1-chloro-2-butene (1-Chloro-2-pentene, β-Ethylallyl chloride) (VI) (18, 19).**—Ninety-three grams (0.783 mole) thionyl chloride was placed in a 200-cc. flask equipped with a dropping funnel and a reflux condenser bearing a calcium chloride tube and brought to a gentle boil on a steam bath. Fifty-seven and two-tenths grams (0.665 mole) 4-methyl-2-butenol (I) was added dropwise over a period of one hour. Gentle heating was continued for four hours after total addition. The reaction mixture was washed several times with successive portions of water and finally with a 5% $NaHCO_3$ solution. The dark brown liquid was dried with anhydrous $CaCl_2$, separated and distilled. The fraction boiling steadily distilled at 98–103°/747 mm. A colorless liquid, darkening rapidly with an increasing pungent odor of evolved HCl.

Yield: 35.2 Gm. (50.7%); d_4^{20} = 0.892; n_D^{25} = 1.4290.

Anal.—Calcd. for C_5H_9Cl: Cl, 33.93. Found: Cl, 32.42, 31.67.

7. **4-Cyclohexyl-1-chloro-2-butene (VII).**—Using the same general procedure as for the preparation of (VI), 62 Gm. (0.52 mole) thionyl chloride and 68.3 Gm. (0.443 mole) 4-cyclohexyl-2-butenol (II) yielded the fraction boiling steadily at 103–108°/14 mm. A colorless liquid darkening rapidly with an increasing pungent odor of evolved HCl.

Yield: 32.9 Gm. (43%); d_4^{20} = 0.964; n_D^{25} = 1.4765.

Anal.—Calcd. for $C_{10}H_{17}Cl$: Cl, 20.54. Found: Cl, 16.97, 16.49.

8. **4-Phenyl-1-chloro-2-butene (VIII).**—Using the same general procedure as for the preparation of (VI), 100 Gm. (0.845 mole) thionyl chloride and 106.4 Gm. (0.72 mole) 4-phenyl-2-butenol (III) yielded the fraction boiling steadily at 107–112°/12 mm. A colorless liquid darkening slowly with an increasing pungent odor of evolved HCl.

Yield: 58.5 Gm. (49%); d_4^{20} = 1.032; n_D^{25} = 1.5365.

Anal.—Calcd. for $C_{10}H_{11}Cl$: Cl, 21.28. Found: Cl, 20.31, 19.23.

9. **4-α-Naphthyl-1-chloro-2-butene (IX).**—Using the same general procedure as for the preparation of (VI), 76.3 Gm. (0.64 mole) thionyl chloride and 107.8 Gm. (0.545 mole) 4-α-naphthyl-2-butenol (IV) yielded the fraction boiling steadily at 162.5–167.5°/7 mm. A pale yellow liquid becoming more yellow on standing with an increasing pungent odor of evolved HCl.

Yield: 39.7 Gm. (33.8%); d_4^{20} = 1.139; n_D^{25} = 1.6158.

Anal.—Calcd. for $C_{14}H_{13}Cl$: Cl, 16.37. Found: Cl, 14.54, 14.45.

10. **4-*o*-Ethoxyphenyl-1-chloro-2-butene (X):** Using the same general procedure as for the preparation of (VI), 38.6 Gm. (0.324 mole) thionyl chloride and 52.9 Gm. (0.276 mole) 4-*o*-ethoxyphenyl-2-butenol (V) yielded the fraction boiling steadily at 120–125°/4 mm. A pale yellow liquid darkening on standing with an increasing pungent odor of evolved HCl.

Yield: 26 Gm. (44.6%); d_4^{20} = 1.077; n_D^{25} = 1.5246.

Anal.—Calcd. for $C_{12}H_{15}OCl$: Cl, 16.84. Found: Cl, 16.59, 16.14.

SUMMARY

1. An evaluation has been made of the utility of 3,4-epoxy-1-butene (butadiene monoxide) as an intermediate in the preparation of 4-substituted-2-butenols and of their chloro analogs.

2. A series of five analogous 4-substituted-2-butenols in which the substituents are methyl-, cyclohexyl-, phenyl-, α-naphthyl-, and *o*-ethoxyphenyl- has been prepared, described, and derivatized as their 3,5-dinitrobenzoates. The derivatives have been analyzed for % N, with satisfactory results. All analogs, except that in which the substituent is methyl-,(2-pentenol, β-ethylallyl alcohol), are new.

3. A series of five analogous 4-substituted-1-chloro-2-butenes in which the substituents are methyl-, cyclohexyl-, phenyl-, α-naphthyl-, and *o*-ethoxyphenyl- has been prepared, described, and analyzed for % Cl, with indicative results. All analogs, except that in which the substituent is methyl-, (1-chloro-2-pentene, β-ethylallyl chloride), are new.

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(5) Pr[illegible]
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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Methionine sulfone
Ketomethionine
4-Diethylamino-2-nitrosophenol
Thyroglobulin
3,3′-Dimethoxybenzidine-5,5′-disulfonic acid
N-(4-azobenzenenaphthyl-1) ethylene diamine
Methoxinine
Guaiacol-4,6-dialdehyde
Guaiacol-4,6-dicarboxylic acid
4-Menthene-3-one
Indole-3-aldehyde
Tryptamine
2-Hexenal
Glucamine
d-Erythrose
d-Threose
Enol phosphopyruvic acid
Piperonylacetonitrile
4-Hexene-2-one
3-Geranylcatechol

Acute Toxicity of Arsenate of Lead in Animals*†

By J. L. VOIGT, L. D. EDWARDS, and C. H. JOHNSON

The oral LD_{50} of lead arsenate in a single dose was determined for several species of animals. For chickens it was calculated that the LD_{50} was 450 mg./Kg. In white rats the LD_{50} of lead arsenate was found to be 825 mg./Kg. For rabbits the LD_{50} was calculated as 125 mg./Kg.

ARSENATE OF lead is a poison used commonly in agriculture as a spray. Because of the possibility of farm animals ingesting lead arsenate residues when feeding on or near vegetation that has been sprayed, much interest has been shown in the toxicity of this compound. Since the quantity of residue ingested at any one time usually is small, much of the work done has dealt only with chronic poisoning. Very few investigators have attempted to determine the size of the single oral dose of lead arsenate which would cause death in higher animals. This paper presents some work done with the latter purpose in view.

Thomas and Shealy (1), in reporting their results with chickens, stated that there appeared to be no definite correlation between the weights of the birds and a lethal dose of lead arsenate. From inspection of their data it seems that 3 to 7 Gm. of the salt would usually kill a chicken, although they they did not state such a conclusion.

McCulloch (2) determined that lead arsenate equivalent to 2 Gm. of arsenic trioxide is required to kill a sheep weighing approximately 40 Kg.

The M. L. D. for rabbits and hares is 200 mg./Kg., according to Chappelier (3). Apparently no other work has been recorded for the acute toxicity of lead arsenate for higher animals.

EXPERIMENTAL

The lead arsenate used had the formula $PbHAsO_4$ and was 200 mesh or less in fineness.

The toxicity was expressed as the LD_{50}, which was calculated by the method of Behrens as described by Burn (4). The rationale of this method was originally proposed by Dragstedt and Lang (5).

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† Abstract of a part of a dissertation presented to the Graduate Council, University of Florida, by J. L. Voigt, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Chickens.—White Leghorn roosters, approximately 2 Kg. in weight, were force fed with the calculated single dose in a capsule. The first symptom of poisoning was a lack of appetite. Upon autopsy of the dead birds it was seen that the crop and proventriculus were inflamed, the gizzard was necrotic, and the small intestine inflamed and hemorrhagic.

Doses of 500 to 5000 mg./Kg. were given to a series of ten birds. All but two birds died within forty days, the survivors having been fed 1000 and 1500 mg./Kg., respectively.

Following these exploratory doses ten chickens were given 1000 mg./Kg. of lead arsenate with ten deaths occurring in seven to fourteen days.

Another ten birds were given 500 mg./Kg. of the compound. Each of these died in eight to eighteen days. The loss in weight and the red cell count were determined on the birds still alive after twelve days with following results:

TABLE I.—RED CELL COUNT AND CHANGE IN WEIGHT IN CHICKENS AFTER FEEDING 500 MG./KG. OF LEAD ARSENATE

Weight When Fed (3/8/45), Kg.	Weight 12 Days Later (3/20/45), Kg.	Red Cell Count	Date of Death
2.23	1.47	1,860,000	3/20/45
2.16	1.36	2,450,000	3/26/45
1.80	1.35	1,600,000	3/23/45
1.72	1.28	1,780,000	3/21/45

The average red cell count for chickens is considered by Scarborough (6) to be 3,440,000, with a normal range of 2,800,000 to 4,500,000.

Ten chickens were given 300 mg./Kg. of lead arsenate. All survived. After thirty-three days the red cell count was only slightly below normal and the weight loss was small.

To another ten chickens 400 mg./Kg. were administered. Three deaths occurred in twelve days while the survivors showed no consistent change in weight or red cell count.

The final ten chickens were given 450 mg./Kg. with four deaths resulting in twelve days. There was some loss in weight among the survivors. By calculation from the data given in Table II it was found that the LD_{50} for chickens is near 450 mg./Kg.

White Rats.—An aqueous suspension of lead arsenate was fed by stomach tube to white rats of both sexes, most of whom weighed between 150 and

TABLE II.—MORTALITY FOR CALCULATING THE LD_{50} OF LEAD ARSENATE IN CHICKENS

Dose, Mg./Kg.	Observed Mortality	Deduced Mortality
1000	10/10	..
500	10/10	..
450	4/10	7/13
400	3/10	3/16
300	0/10	..

230 Gm. An exploratory test was carried out by feeding ten rats doses varying from 175 to 425 mg/Kg. No toxic symptoms developed.

Ten rats, given 500 mg./Kg., exhibited diarrhea and lack of appetite for a few days; one death occurred after twenty-five days. In another group, ten rats were given 600 mg./Kg. with two deaths resulting within three weeks. A dose of 700 mg./Kg. caused three deaths in ten animals in fifteen to twenty days; upon examination of the dead rats symptoms of irritation of the intestinal tract were observed. In another group four rats out of ten died from 800 mg./Kg. within six days; the others survived. In the next series of ten rats which received 900 mg./Kg., five deaths occurred in six to eighteen days. The final group received 1000 mg./Kg. and seven out of ten died in two to twelve days. All the dead rats showed similar indications of arsenic poisoning on post-mortem examination. The LD_{50} for lead arsenate in white rats was calculated from the data given in Table III as approximately 825 mg./Kg.

TABLE III.—MORTALITY FOR CALCULATING THE LD_{50} OF LEAD ARSENATE IN WHITE RATS

Dose, Mg./Kg.	Observed Mortality	Deduced Mortality
1000	7/10	22/25
900	5/10	15/23
800	4/10	10/24
700	3/10	6/27
600	2/10	3/32
500	1/10	1/39

Rabbits.—The lead arsenate was administered to rabbits of both sexes by stomach tube in the same way as it was given to the white rats. These animals were obtained from various sources and weighed between 1 and 2 Kg.

Ten rabbits were given doses varying between 100 and 325 mg./Kg. with the result that all died within six days. Post-mortem examinations showed the characteristic picture of lead arsenate poisoning: inflammation, necrosis, and clotting of blood in the gastrointestinal tract.

Eight more rabbits were given doses of 5 to 100 mg./Kg. The animals receiving 25, 50, and 100 mg./Kg. died, while the one receiving 75 mg./Kg. eventually recovered after exhibiting toxic symptoms.

After these preliminary trials ten rabbits were fed 20 mg./Kg.; one animal died ten days later. The next group of ten animals received 40 mg./Kg.; four days later one rabbit died. Fifty mg./Kg. were fed to each of seven animals in another group, with one death being recorded four days later. Sixteen rabbits were given 100 mg./Kg. with six deaths within three days. Ten more rabbits were fed 150 mg./Kg. and four died in four to twelve days. In the final series ten rabbits received 200 mg./Kg. All died within two days with severe necrosis and profuse bleeding in the stomach. The LD_{50} for lead arsenate in rabbits was calculated as approximately 125 mg./Kg. from the data in Table IV.

TABLE IV.—MORTALITY FOR CALCULATING THE LD_{50} OF LEAD ARSENATE IN RABBITS

Dose, Mg./Kg.	Observed Mortality	Deduced Mortality
200	10/10	...
150	4/10	13/19
100	6/16	9/25
50	1/7	3/25
40	1/10	2/33
20	1/10	1/41

SUMMARY

1. From the results of feeding lead arsenate to 50 chickens in doses varying from 300 to 1000 mg./Kg. it was calculated that the LD_{50} is near 450 mg./Kg. for these fowl. All chickens receiving doses of 500 and 1000 mg./Kg. died. This dose compares with the fatal dose of 60 to 150 mg./Kg. for arsenic trioxide in chickens as tabulated by Heffter (7).

2. The weight and the red cell count of chickens decreased after ingestion of 500 mg./Kg. of lead arsenate.

3. The LD_{50} for lead arsenate in white rats was calculated as approximately 825 mg./Kg. after feeding doses of 500 to 1000 mg./Kg. to 60 animals. After the largest dose seven out of ten animals died.

4. Doses of 20 to 200 mg./Kg. of lead arsenate were fed to 63 rabbits and the LD_{50} calculated therefrom as about 125 mg./Kg. A dose of 200 mg./Kg. killed ten rabbits out of ten. Arsenic trioxide has been reported as fatal by Heffter (7) in doses of 10 to 30 mg./Kg.

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The Action of Intestinal Enzymes upon Cellulose Acetate Phthalate and Butyl Stearate Enteric-Coated Tablets*†

By CHARLES W. BAUER‡ and PETER E. MASUCCI

In considering the disintegration of enteric coatings of tablets it is essential to differentiate between intestinal contents and intestinal secretions. Enteric-coated tablets disintegrate in the intestinal contents which are seldom, if ever, alkaline. The disintegration of cellulose acetate phthalate and butyl stearate is due to the hydrolytic effect of the intestinal esterases and not, as is commonly believed, upon the alkalinity of the intestines.

It is generally agreed that more and more medicines should be enteric coated, both for the avoidance of gastric distress and for the assurance of therapeutic action. The preparation of a satisfactory enteric coating is not a simple procedure, nor is its evaluation a simple and easy task. Unfortunately many of the enteric coatings now on the market have not been properly evaluated. It seems, then, that if we are going to be called on for additional enteric-coated preparations, further work in this field of investigation is desirable.

The demands, as well as the restrictions, that pharmacy has placed upon the materials that are used to make enteric preparations are fairly well known. Very briefly, the specifications call for a nontoxic and physiologically inert material that must withstand the action of the gastric juice, but must disintegrate in the intestines.

The criticism that should be made about enteric preparations is directed against the tests that are used to evaluate them. It is assumed that the coating that is used is nontoxic and is physiologically inert, for these qualifications can be sufficiently tested. Attention should be devoted to the other tests, especially to those that involve the use of acids and alkalies. A typical report on the analysis of an enteric coating states that it is resistant to 0.3 per cent hydrochloric acid at body temperature for four and one-half hours, and that it is completely broken down in a 0.5 per cent sodium bicarbonate solution in one and one-half hours. On the strength of this analysis the substance is said to be satisfactory. Literature citations and experimental evidence presented in this paper point out that the effectiveness of an enteric coating cannot be evaluated upon its disintegration in a solution of sodium bicarbonate.

Certain coatings already known to be unsatisfactory were not studied in this work. Keratin was tested by Bukey and Rhodes (1), and was found to be highly efficient when properly applied, but very difficult of proper and even application. Formaldehyde-gelatin was tested by Goorley and Lee (2), who found it undesirable because upon aging it became too hard and would not disintegrate. Bukey and Brew (3) found tolu to be of very little value because it, too, would fail to disintegrate if not freshly made. Worton, Kempf, Burrin, and Bibbins (4) found collodion to be of no use at all because of its insolubility in the intestinal juices. These authors found agar and elm bark also to be unsatisfactory, because the time of disintegration was too variable.

Cellulose acetate phthalate has received considerable publicity as a very satisfactory enteric coating. The literature (5) indicates that this substance does not disintegrate in the stomach but does disintegrate in the small intestine. However, the reasons given for the decomposition of cellulose acetate phthalate seem to be erroneous. It is true

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† This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Peter E. Masucci in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
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that it does not disintegrate in 0.3 per cent hydrochloric acid in four hours and does disintegrate in a 0.5 per cent sodium bicarbonate solution in one-half hour. But its behavior in such *in vitro* tests cannot be assumed to explain its action *in vivo*.

The intestinal juice does not resemble a 0.5 per cent solution of sodium bicarbonate. Then why is this test made? It is made because of a widespread erroneous belief that the contents of the intestines are alkaline. This error has crept into pharmaceutical and medical literature and will require some time to be corrected. There is ample evidence to show that the contents of the intestines must be distinguished from the secretions of the intestines, and are seldom, if ever, alkaline. McClendon (6) has shown that the contents of the intestines are acid, the *p*H rising in the distal levels, but even in the ileum the contents are rarely above neutral. A good review of this subject has been given by Verzar (7).

TABLE I.—EFFECT OF PANCREATIC ESTERASE ON CELLULOSE ACETATE PHTHALATE AT 37° FOR ONE HOUR SHOWN BY THE QUANTITY OF 0.1 *N* SODIUM HYDROXIDE TO NEUTRALIZE THE REACTION MIXTURE

	Flask 1 (Control)	Flask 2 (Control)	Flask 3 (Control)	Flask 4
Calcium chloride 2%, cc.	1	1	1	1
Cellulose acetate phthalate, Gm.	1	..	1	1
Pancreatin U. S. P., Gm.	..	1	1 (boiled)	1
Distilled water, cc.	25	25	25	25
0.1 *N* sodium hydroxide, cc.	11.2	6.8	18.5	28.1

EXPERIMENTAL

Cellulose Acetate Phthalate as an Enteric Coating.—Cellulose acetate phthalate is an organic ester that should be acted upon by the esterases. To determine the effect of esterases upon cellulose acetate phthalate experiments were made in which Pancreatin U. S. P. was used as the source of esterases for this determination.

Four Erlenmeyer flasks of 125-cc. capacity were designated as flask 1, flask 2, flask 3, and flask 4. Into flask 1, 1 cc. of a 2% solution of calcium chloride, 1 Gm. of cellulose acetate phthalate, and 25 cc. of distilled water were placed. This flask was placed in a constant temperature bath maintained at 37° for one hour. After one hour the mixture was titrated with 0.1 *N* sodium hydroxide using thymol blue as the indicator.

Into flask 2, 1 cc. of a 2% solution of calcium chloride, 1 Gm. of Pancreatin U. S. P., and 25 cc. of distilled water were placed. The same procedure was followed with this mixture as has been described for that in flask 1.

In flask 3, 1 cc. of a 2% solution of calcium chloride, 1 Gm. of Pancreatin U. S. P., and 25 cc. of distilled water were added and were brought to the boiling point to destroy the enzymes in the Pancreatin. The mixture was then cooled to room temperature and 1 Gm. of cellulose acetate phthalate was added. This mixture was then placed in a constant temperature bath at 37° for one hour and was then titrated with 0.1 *N* sodium hydroxide as above.

Into flask 4, 1 cc. of a 2% solution of calcium chloride, 1 Gm. of cellulose acetate phthalate, 1 Gm. of Pancreatin U. S. P., and 25 cc. of distilled water were placed. The same procedure was carried out here as with flasks 1 and 2. The titration showed that flask 4 required more 0.1 *N* sodium hydroxide than was required in the other three flasks. This seems to indicate that the cellulose acetate phthalate was acted upon by the pancreatic esterases. The hydrolytic effect of the esterases upon the cellulose acetate phthalate produced acids, which accounts for the use of a greater amount of 0.1 *N* sodium hydroxide for neutralization. The experimental evidence of these findings may be seen in Table I.

In the next step of the investigation, cellulose acetate phthalate was applied as an enteric coating to 5-gr. sodium salicylate tablets. The formula for the cellulose acetate phthalate mixture found most satisfactory was introduced by Abbot and Allport (8). This formula is as follows:

Cellulose Acetate Phthalate.....	5.0 parts
Ethyl Acetate.....	47.5 parts
Alcohol 95%.....	47.5 parts

A satisfactory method of coating these tablets was to place them in a revolving copper drum and to apply the coating in the form of a fine spray from an atomizer. At the same time a blast of hot air was directed into the drum to hasten the evaporation of the solvent and the drying of the coating. After the tablets had been well coated, they were weighed to determine how much coating was on each tablet. The coating was found to average 0.4 gr. on each tablet. The test used to determine the quality of the coating consisted in allowing the tablets to stand in a dilute solution of ferric chloride for two or

three hours. If the coating contained "pinholes" the sodium salicylate would soon manifest itself by a dark coloration of the solution. If the tablets withstood this test, they were considered to be satisfactory for the next step in the investigation.

The next step was to submit the tablets to *in vitro* analysis not only in the manner in which it is ordinarily done, but also in a new test, based upon the fact that the intestinal contents are neutral or even acid. The following solutions were prepared for this part of the analysis.

1. *Artificial gastric juice* (9):

Pepsin N. F.	0.5	Gm.
Hydrochloric Acid	0.7	Gm.
Lactic Acid	0.2	Gm.
Distilled Water	100.0	cc.

2. *Artificial intestinal juice (older type)* (10):

Pancreatin U. S. P.	0.1	Gm.
Sodium Bicarbonate	1.0	Gm.
Distilled Water	100.0	cc.

3. *Artificial intestinal juice (newer type)* (11):

Pancreatin U. S. P.	1.0	Gm.
Calcium Chloride Solution (1%)	1.0	cc.
M/5 Potassium Biphosphate.	25.0	cc.
M/5 Sodium Hydroxide	11.8	cc.
Distilled Water	100.0	cc.

These solutions were maintained at 37° by means of a constant temperature bath. Five coated tablets were placed in the artificial gastric juice to determine the length of time it would take for the gastric juice to act upon the coating. The coated tablets not only remained intact for four and one-half hours but showed no signs of disintegration in seven days. The artificial gastric juice was kept in motion by a motor-driven stirrer during the entire period of observation.

Five of the coated tablets were placed in the artificial intestinal juice (older type) at a *p*H of 8.5. This mixture also was kept at a constant temperature of 37°; and it, too, was stirred mechanically. All five tablets disintegrated within fifty minutes.

Finally five of the coated tablets were placed in the artificial intestinal juice (newer type). This juice was prepared in accordance with the new theory that the contents of the intestines are not alkaline. It had a *p*H of 6.9. The same conditions prevailed here as with the other two solutions; that is, the temperature was maintained at 37°, and continual motion was obtained by means of a mechanical stirrer. Once again the length of time was noted for disintegration. All five tablets first showed signs of rupture after from seventy to seventy-five minutes. Thus it was seen that the enteric-coated tablets stood up remarkably well in the artificial gastric juice but were acted upon by both types of artificial intestinal juice. The newer type of artificial intestinal juice, even though it was started at a *p*H of 6.9 and certainly did not become more alkaline as the digestion took place, brought on disintegration of the tablets because of the enzyme action, and not because of the alkalinity of the solution. Repeated trials gave similar results. It was further observed that a 1% solution of sodium bicarbonate without any Pancreatin caused the tablets to disintegrate in one hour, while the buffered solution without the Pancreatin had no effect upon the tablets.

Since the enteric coating of cellulose acetate phthalate was seen to be acted upon by pancreatic esterases when the test was made *in vitro*, the next step was to determine how it would act *in vivo*. Sodium salicylate was the drug chosen for this purpose since it has been shown by Lazenski and Diver (12) that this drug is readily detected in the urine by ferric chloride. The absence of a reaction indicates that no disintegration of enteric-coated sodium salicylate has taken place. It is known that 5 drops of a 1% aqueous solution of sodium salicylate gives, with ten drops of 10% solution of ferric chloride, a violet color which is permanent for more than fifteen minutes. Now if a tablet of sodium salicylate is enteric coated and is then swallowed, there will be no sodium salicylate found in the urine until the coating has disintegrated and the salicylate has been absorbed and has reached the urine. If a positive test is obtained in less than two or three hours, it probably indicates that the enteric coating has been acted upon in the stomach. However, if a positive test is not obtained until five or six hours after the tablet has been ingested, it usually indicates that the coating has withstood the action of the gastric juice and has disintegrated in the intestine. If no test at all is obtained, then the coating has not disintegrated and the tablet is usually found in the feces intact.

Two 5-gr. tablets of sodium salicylate coated with cellulose acetate phthalate were taken orally with two tumblerfuls of water. The time of administration was recorded, and at hourly intervals the urine was tested for salicylates in the manner described. For controls a group of normal adults were given two 5-gr. tablets of sodium salicylate. The bladder was emptied before the tablets were taken and samples of urine were then assayed at hourly intervals. In 5 subjects a positive test was obtained in one hour while in one subject the test was only moderately positive in one hour but was very strong at the end of two hours. It will be noted that the cellulose acetate phthalate tablets did not give a positive test in less than four hours. It will also be observed that the majority of the subjects gave positive tests in a period of six hours. Hence, it may be assumed that the cellulose acetate phthalate readily disintegrates in the small intestine. Those tablets requiring more than seven hours for a positive test may have reached the colon before suffering disintegration. Since cellulose acetate phthalate is acted upon by pancreatic esterases, and since the intestinal contents are not alkaline, it appears that this ester suffers disintegration in the intestines because it serves as a substrate for intestinal esterases. The experimental results are summarized in Table II.

TABLE II.—TIME REQUIRED FOR SODIUM SALICYLATE TO APPEAR IN THE URINE WHEN TWO CELLULOSE ACETATE PHTHALATE ENTERIC-COATED SODIUM SALICYLATE TABLETS WERE INGESTED

No. of Adult Subjects	No. Hours Required for a Positive Test									
	1	2	3	4	5	6	7	8	9	10
5 uncoated tablets	+	..	..	..	..	..	..	..	..	..
1 uncoated tablet	—	+	..	..	..	..	..	..	..	..
1	—	—	—	+	..	..	..	..	..	..
2	—	—	—	—	+	..	..	..	..	..
18	—	—	—	—	—	+	..	..	..	..
2	—	—	—	—	—	—	+	..	..	..
2	—	—	—	—	—	—	—	+	..	..
1	—	—	—	—	—	—	—	—	+	..
1	—	—	—	—	—	—	—	—	—	+

TABLE III.—TIME REQUIRED FOR SODIUM SALICYLATE TO APPEAR IN THE URINE WHEN TWO BUTYL STEARATE ENTERIC-COATED 5-GR. SODIUM SALICYLATE TABLETS WERE INGESTED

No. of Subjects	No. Hours Required for a Positive Test								
	1	2	3	4	5	6	7	8	9
5 uncoated tablets	+	..	..	..	..	..	..	..	..
1	—	—	—	—	+	..	..	..	..
6	—	—	—	—	—	+	..	..	..
5	—	—	—	—	—	—	+	..	..
4	—	—	—	—	—	—	—	+	..
4	—	—	—	—	—	—	—	—	+

Butyl Stearate as an Enteric Coating.—The ester, butyl stearate, when used in the same manner as cellulose acetate phthalate, as an enteric coating for sodium salicylate tablets, required a longer period of time for the salicylate to appear in the urine. One reason for this longer period of time was undoubtedly due to the fact that twice as much butyl stearate than cellulose acetate phthalate had to be applied in order to get an impervious coating. A further study of the effectiveness of butyl stearate as an enteric coating was made by applying it to barium sulfate tablets and by observing the place of distingegration by means of X-ray visualization.

Five-grain tablets of sodium salicylate as well as 5-gr. tablets of barium sulfate were coated with the following mixture:

Butyl Stearate	50 parts
Shellac	100 parts
Alcohol	200 parts

The tablets were placed in a revolving drum; the butyl stearate mixture was applied from a beaker; talc was used as the running powder, and air was directed on the revolving tablets to help dry the coating.

The increase in weight of each barium sulfate tablet was 0.8 gr. Only one barium sulfate enteric-coated tablet was given to each subject.

It was found that the barium sulfate enteric-coated tablets passed through the jejunum before they uffered disintegration. About 50% of the trials showed disintegration to take place in the lower ileum and about an equal number reached the caecum; but not all of the tablets that reached the caecum disintegrated within eight hours. It was impractical to hold the subjects for a longer period of time. While most drugs are not absorbed from the large intestine it can be shown that sodium salicylate will give a urine test when administered rectally. Hence, some of the positive tests one may obtain when sodium salicylate is used to test enteric coatings fail to give the position where the distingegration takes place. It seems that butyl stearate may not serve as a useful enteric coating when it is applied in the manner indicated in this paper, if the drug on which it is applied is intended for the circulation; it may, however, be useful if the drug is not intended for the circulation but is used for its action upon organisms in the lower ileum or colon.

In all cases the tablet was given within one hour after breakfast. The majority of the tablets moved out of the stomach in less than two hours. All sub-

TABLE IV.—TIME REQUIRED FOR ONE BARIUM SULFATE TABLET TO DISINTEGRATE IN THE INTESTINAL TRACT WHEN COATED WITH BUTYL STEARATE AS SEEN BY X-RAY VISUALIZATION

No. of Subjects	Place of Disintegration	No. Hours After Ingestion				
		4	5	6	7	8
2	Ileum	—	+	..	..	..
3	Ileum	—	—	+	..	..
5	Ileum	—	—	—	+	..
2	Colon	—	—	—	—	+
5	In colon but no disintegration	—	—	—	—	—
1	In stomach but no disintegration	—	—	—	—	—

jects had a luncheon before the tests were completed.

The results obtained with butyl stearate enteric-coated sodium salicylate tablets may be seen in Table III.

The results obtained with butyl stearate enteric-coated barium sulfate tablets observed by means of X-ray visualization may be seen in Table IV.

SUMMARY

1. One interested in the disintegration of enteric coatings must differentiate between intestinal secretions and intestinal contents.

2. Enteric-coated tablets disintegrate in the intestinal contents, and not in the intestinal secretions.

3. The *p*H of the intestinal contents is seldom, if ever, alkaline.

4. The disintegration of enteric coatings like cellulose acetate phthalate in the intestinal contents, which are on the acid side, is the result of the hydrolytic action of the intestinal esterases.

5. Cellulose acetate phthalate is a satisfactory enteric coating that for the most part disintegrates in the jejunum.

6. Butyl stearate seems not to disintegrate in the jejunum as seen by X-ray visualization, but does show disintegration in the ileum and colon. This slowness in disintegration may be due to the fact that a heavier coating of butyl stearate than cellulose acetate phthalate was used. Some of the butyl stearate enteric-coated tablets failed to disintegrate in eight hours.

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Book Reviews

Pharmacology and Pharmacotherapeutics for Dentists. Third Edition. By WILLIAM H. O. MCGEHEE and MELVIN W. GREEN. The Blakiston Company, Philadelphia, Pa., 1947. xx + 536 pp. 16 x 23.5 cm. Price $6.50.

This excellent pharmacology textbook for dental students has been extensively revised for this edition. The authors have recognized the rapid development of new drugs and the changes in our knowledge of various drug actions. Among the parts brought up to date, especially from the viewpoint of dental practice, are those on the sulfonamides, vitamins, hormones, and antibiotics.

The book is divided into eight sections, each covering a particular part of the general field. The first section is an introduction to the basic principles of pharmacology, pharmacy, and prescription writing. The other sections cover the locally acting drugs, especially those used in the mouth or on the teeth, the systemically acting drugs, and the miscellaneous substances such as the metals, specifics, vitamins, and hormones. The appendix includes sections on the symptoms and treatment of acute poisoning, various useful tables, and a comprehensive dental formulary.

While the authors have covered the entire field of pharmacology, they have emphasized the drugs useful to the dentist. The drugs which are considered, in greatest detail include antiseptics, disinfectants, irritants, caustics, bleaching agents, dentifrices, local anesthetics, central stimulants, anesthetics, and analgesics.

Some of the material appears to be superfluous from the standpoint of the dental student. For example, the section on the theories of drug action is too short to be of value and just long enough to be confusing to the student. The systemic effects of procaine do not appear to be adequately presented, especially in view of the wide usage of this drug. The statement that the action of ephedrine is to inhibit epinephrine oxidase is not correct.

This book is well written and should be very useful both to the student and to the practicing dentist.—RAYMOND P. AHLQUIST.

Estimation of the Vitamins. Edited by W. J. DANN and G. HOWARD SATTERFIELD. The Jaques Cattell Press, Lancaster, Pa., 1947. 531 pp. 16 x 25 cm. Price $6.50.

Methods of Vitamin Assay. Prepared and edited by the ASSOCIATION OF VITAMIN CHEMISTS. Interscience Publishers, Inc., New York, 1947. xviii + 189 pp. 15 x 23 cm. Price $3.50.

The biological assay of the vitamins is as old as knowledge of their existence. The isolation and chemical characterization of the vitamins stimulated publication in this important assay field so that during the past fifteen years thousands of publications have appeared. Because complex foodstuffs are the source of the vitamins, their separation and measurement are not always easy. The problem is further complicated by the fact that many of the vitamins occur in nature in more than one chemical state. Vitamin A often occurs as esters. Carotene has vitamin A activity, but there is more than one carotene and they do not have equal physiological activity. Thiamin can occur as co-carboxylase; nicotinic acid can also exist as the amide. Ascorbic acid sometimes is present as dehydro-ascorbic acid—and so on it goes.

Vitamins may be assayed biologically by preventive and curative techniques. They may be determined by a number of chemical techniques—colorimetric methods, ultraviolet light absorption methods, simple titrations, gasometric methods and others. In addition they are often determined by microbiological methods. Even a human biological assay, based in part on chemical methods, has been proposed.

Confronted with the myriad of papers in the literature, many of them dealing with specific methods for special purposes, the average worker entering the field is almost hopelessly confused. To coordinate the literature and standardize the vitamin methods is the purpose of these two useful volumes.

The first of these volumes, Volume XII of the Biological Symposia series, represents the review type approach. Each of the 29 chapters is written by an expert. E. M. Nelson, Esmond E. Snell, C. A. Elvehjem, John V. Scudi, Charles E. Bills, H. J. Almquist, and many other such famous names grace the pages of this book. In general, separate chapters are written on bioassay, physico-chemical methods, and microbiological methods for each of the vitamins. All of the well-characterized vitamins are included. Each chapter contains its bibliography.

The second book is quite unique and is more of a manual than a review treatise. It was prepared under the jurisdiction of the Association of Vitamin Chemists. Only such methods have been included which are in general use as determined by committees of specialists. Emphasis has been placed on those details on which the usefulness and precision of a method depend. Extensive notes have been added. Only where more or less essential are literature references cited. These two books are natural complements to each other and certainly they will soon become the "bibles" of workers in the vitamin field.

The Water-Soluble Gums, by C. L. MANTELL. Reinhold Publishing Corporation, 330 W. 42nd St., New York 18, N. Y. v + 279 pp. 15 x 23 cm. Price $6.

For many years practice and art were ahead of science in the gum field; however, the author has in this book attempted to coordinate the information relating to the water-dispersible products derived from a wide variety of plants and known as gums. In this he has succeeded very well because he has brought together into 16 chapters all of the well-known, and not so well-known, gums and has classified them into groups with similar properties.

In each of these groups of gums the origin, physical, and chemical properties are discussed thoroughly. The author also includes an excellent chapter on Specifications, Identification, and Testing of all of the more well-known gums giving description, physical properties, tests for identity and purity, and 37 methods for the testing of the various constituents of the gums.

Also included is an extensive table of the Nature Designation of the Gums, consisting of name, place of origin, type, and present-day classification; a botanical index giving the botanical name and page reference of all gums discussed in the book; and an index of all authors cited in references.

This book is well written, interesting, and technical only to the point of necessity and should be of value to anyone interested or working in the field of "gum technology."

Fatty Acids: Their Chemistry and Physical Properties, by KLARE S. MARKLEY. Interscience Publishers, Inc., New York, 1947. x + 668 pp. 15 x 23 cm. Price $10.

The author of this excellent treatise has brought together the essential literature on the fatty acids in an organized fashion. The book is divided into six sections containing a total of 23 chapters. The sections are entitled: The Nature and History of Fats and Waxes, Classification and Structure of Fatty Acids, Physical Properties of the Fatty Acids, Chemical Reactions of the Fatty Acids, Syntheses of Fatty Acids, and Isolation of Fatty Acids.

The work is thoroughly modern in its outlook with considerable of the physical-chemical approach. Careful and discriminating documentation can be seen throughout. The author shows evidence of having tried to select the most correct physical properties from data which often extend back over about fifty years and frequently covered periods when the methodology was not so advanced.

Fatty acids play such an important role in pharmacy, that no research-minded pharmaceutical scientist should be without this reference.

jects had a luncheon before the tests were completed.

The results obtained with butyl stearate enteric-coated sodium salicylate tablets may be seen in Table III.

The results obtained with butyl stearate enteric-coated barium sulfate tablets observed by means of X-ray visualization may be seen in Table IV.

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Book Reviews

Pharmacology and Pharmacotherapeutics for Dentists. Third Edition. By WILLIAM H. O. MCGEHEE and MELVIN W. GREEN. The Blakiston Company, Philadelphia, Pa., 1947. xx + 536 pp. 16 x 23.5 cm. Price $6.50.

This excellent pharmacology textbook for dental students has been extensively revised for this edition. The authors have recognized the rapid development of new drugs and the changes in our knowledge of various drug actions. Among the parts brought up to date, especially from the viewpoint of dental practice, are those on the sulfonamides, vitamins, hormones, and antibiotics.

The book is divided into eight sections, each covering a particular part of the general field. The first section is an introduction to the basic principles of pharmacology, pharmacy, and prescription writing. The other sections cover the locally acting drugs, especially those used in the mouth or on the teeth, the systemically acting drugs, and the miscellaneous substances such as the metals, specifics, vitamins, and hormones. The appendix includes sections on the symptoms and treatment of acute poisoning, various useful tables, and a comprehensive dental formulary.

While the authors have covered the entire field of pharmacology, they have emphasized the drugs useful to the dentist. The drugs which are considered, in greatest detail include antiseptics, disinfectants, irritants, caustics, bleaching agents, dentifrices, local anesthetics, central stimulants, anesthetics, and analgesics.

Some of the material appears to be superfluous from the standpoint of the dental student. For example, the section on the theories of drug action is too short to be of value and just long enough to be confusing to the student. The systemic effects of procaine do not appear to be adequately presented, especially in view of the wide usage of this drug. The statement that the action of ephedrine is to inhibit epinephrine oxidase is not correct.

This book is well written and should be very useful both to the student and to the practicing dentist.—RAYMOND P. AHLQUIST.

Estimation of the Vitamins. Edited by W. J. DANN and G. HOWARD SATTERFIELD. The Jaques Cattell Press, Lancaster, Pa., 1947. 531 pp. 16 x 25 cm. Price $6.50.

Methods of Vitamin Assay. Prepared and edited by the ASSOCIATION OF VITAMIN CHEMISTS. Interscience Publishers, Inc., New York, 1947. xviii + 189 pp. 15 x 23 cm. Price $3.50.

The biological assay of the vitamins is as old as knowledge of their existence. The isolation and chemical characterization of the vitamins stimulated publication in this important assay field so that during the past fifteen years thousands of publications have appeared. Because complex foodstuffs are the source of the vitamins, their separation and measurement are not always easy. The problem is further complicated by the fact that many of the vitamins occur in nature in more than one chemical state. Vitamin A often occurs as esters. Carotene has vitamin A activity, but there is more than one carotene and they do not have equal physiological activity. Thiamin can occur as co-carboxylase; nicotinic acid can also exist as the amide. Ascorbic acid sometimes is present as dehydro-ascorbic acid—and so on it goes.

Vitamins may be assayed biologically by preventive and curative techniques. They may be determined by a number of chemical techniques—colorimetric methods, ultraviolet light absorption methods, simple titrations, gasometric methods and others. In addition they are often determined by microbiological methods. Even a human biological assay, based in part on chemical methods, has been proposed.

Confronted with the myriad of papers in the literature, many of them dealing with specific methods for special purposes, the average worker entering the field is almost hopelessly confused. To coordinate the literature and standardize the vitamin methods is the purpose of these two useful volumes.

The first of these volumes, Volume XII of the Biological Symposia series, represents the review type approach. Each of the 29 chapters is written by an expert. E. M. Nelson, Esmond E. Snell, C. A. Elvehjem, John V. Scudi, Charles E. Bills, H. J. Almquist, and many other such famous names grace the pages of this book. In general, separate chapters are written on bioassay, physico-chemical methods, and microbiological methods for each of the vitamins. All of the well-characterized vitamins are included. Each chapter contains its bibliography.

The second book is quite unique and is more of a manual than a review treatise. It was prepared under the jurisdiction of the Association of Vitamin Chemists. Only such methods have been included which are in general use as determined by committees of specialists. Emphasis has been placed on those details on which the usefulness and precision of a method depend. Extensive notes have been added. Only where more or less essential are literature references cited. These two books are natural complements to each other and certainly they will soon become the "bibles" of workers in the vitamin field.

The Water-Soluble Gums, by C. L. MANTELL. Reinhold Publishing Corporation, 330 W. 42nd St., New York 18, N. Y. v + 279 pp. 15 x 23 cm. Price $6.

For many years practice and art were ahead of science in the gum field; however, the author has in this book attempted to coordinate the information relating to the water-dispersible products derived from a wide variety of plants and known as gums. In this he has succeeded very well because he has brought together into 16 chapters all of the well-known, and not so well-known, gums and has classified them into groups with similar properties.

In each of these groups of gums the origin, physical, and chemical properties are discussed thoroughly. The author also includes an excellent chapter on Specifications, Identification, and Testing of all of the more well-known gums giving description, physical properties, tests for identity and purity, and 37 methods for the testing of the various constituents of the gums.

Also included is an extensive table of the Nature Designation of the Gums, consisting of name, place of origin, type, and present-day classification; a botanical index giving the botanical name and page reference of all gums discussed in the book; and an index of all authors cited in references.

This book is well written, interesting, and technical only to the point of necessity and should be of value to anyone interested or working in the field of "gum technology."

Fatty Acids: Their Chemistry and Physical Properties, by KLARE S. MARKLEY. Interscience Publishers, Inc., New York, 1947. x + 668 pp. 15 x 23 cm. Price $10.

The author of this excellent treatise has brought together the essential literature on the fatty acids in an organized fashion. The book is divided into six sections containing a total of 23 chapters. The sections are entitled: The Nature and History of Fats and Waxes, Classification and Structure of Fatty Acids, Physical Properties of the Fatty Acids, Chemical Reactions of the Fatty Acids, Syntheses of Fatty Acids, and Isolation of Fatty Acids.

The work is thoroughly modern in its outlook with considerable of the physical-chemical approach. Careful and discriminating documentation can be seen throughout. The author shows evidence of having tried to select the most correct physical properties from data which often extend back over about fifty years and frequently covered periods when the methodology was not so advanced.

Fatty acids play such an important role in pharmacy, that no research-minded pharmaceutical scientist should be without this reference.

Encyclopedia of Chemical Technology. Volume I. Edited by RAYMOND E. KIRK and DONALD F. OTHMER. In 10 volumes. The Interscience Encyclopedia, Inc., New York, 1947. xxiv + 981 pp. 18 x 26.5 cm. Price $20.

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Note

Viburnum Studies. XIV. A Note on the Variability of Potency of Commercial Viburnum Preparations*

By A. B. SLOANE, A. R. LATVEN, and JAMES C. MUNCH

IN CONNECTION with our studies on Viburnum, methods of bioassay were proposed in an earlier report (1). At that time we were using the isolated guinea-pig uterus, measuring antagonism to U. S. P. Standard Powdered Posterior Pituitary; also neutralizing the depressor action of Viburnum on anesthetized dogs with U. S. P. Reference Standard Epinephrine. Further research indicated certain advantages in the use of the isolated uterus of the rat, and comparison of the depressor potency of Viburnum with the depressor potency of papaverine HCl on the same uterine strip (2).

Using the isolated rat uterine strip, the quantities of papaverine HCl and of Viburnum required to produce in each instance a 50% decrease in amplitude of spontaneous contractions are estimated. On many tissues only a single dose of Viburnum produces quantitative responses, although some sedation is present after later doses. Papaverine sedation is usually reproducible before the administration of Viburnum; after Viburnum, papaverine tends to become more effective as a uterine sedative.

Using this method, we assayed a large number of commercial Viburnum prunifolium crude barks, also powdered extracts prepared from commercial barks under commercial conditions. Our studies on the barks indicated that a tentative standard might be established at a level of 500 γ of papaverine HCl corresponding to 1 Gm. of crude bark, when assays were made on an alcoholic extract prepared in accordance with a method in N. F. VII. Since the powdered extract represents four times the strength of the drug Viburnum in commercial practice, a tentative standard of 2000 γ of papaverine HCl/Gm. was established in this laboratory as a potency requirement for the powdered extract.

We have assayed a number of commercial Viburnum barks, many of which were identified pharmacognostically and found to be authentic by Dr. Heber W. Youngken. Many of these samples showed potencies of 500 γ/Gm. of bark. However, we have noted some samples showing much lower activities. As indicative of the current activity, the last seven samples tested have shown potencies, arranged in order of increasing strength, of 450, 500, 1035, 1125, 1565, 2335, and 2500 γ/Gm. of Viburnum bark. This is indicative of the trend of activities for Viburnum barks submitted for assay during 1947. Our laboratory test on samples submitted in 1944 and 1945 showed very few samples with a potency greater than 500 γ/Gm., and none over 1000 γ/Gm.

Similarly our early assays of the potency of the powdered extract of Viburnum indicated activities around 500 to 2000 γ/Gm. The improvement of potency of the powdered extracts has not been as noticeable as in the case of the crude drugs, submitted to us for bioassay. The last five samples of powdered extract submitted during 1947 for bioassay have shown values, arranged in order of progression, of 200, 1100, 2500, 2650, and 3000 γ/Gm. In some instances we had analyzed the crude Viburnum as well as the powdered extract prepared therefrom, and found that there was no consistent relationship in potency between the two. In fact some of the extracts were weaker than the crude drugs from which they had been prepared, when both samples were assayed on the same date. We are now starting detailed research in an attempt to learn the cause for such capricious results, and to determine the effect of various factors on the potency of powdered extracts prepared from the same crude drug on a laboratory and on a commercial scale.

CONCLUSIONS

1. The bioassay of Viburnum preparations against papaverine HCl for sedation of the isolated rat uterus has proved satisfactory.
2. As tentative standards 1 Gm. of authentic Viburnum prunifolium bark should correspond in sedative potency to 500 γ of papaverine HCl, and 1 Gm. powdered extract to 2000 γ.

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* Received Sept. 30, 1947 from the Munch Research Laboratories, Upper Darby, Pa.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Enhancement of Penicillin Effectiveness by Traces of Cobalt*

By LOUIS A. STRAIT, JEAN DUFRENOY, and ROBERTSON PRATT, with the Laboratory Assistance of VIRGINIA LAMB†

Addition of trace amounts of cobalt to the nutrient agar in test plates increases approximately four- to eightfold the effectiveness of relatively dilute penicillin solutions in producing inhibition zones on plates seeded with *Staphylococcus aureus* and decreases to approximately one-third or less, depending upon the test organism that is used, the minimum or threshold concentration of penicillin that can be detected by the cylinder-plate technic.

DURING the course of studies of the cytochemical action of penicillin on bacteria (1, 2) and of the interrelationship between the biological and the physical phenomena involved in the assay of penicillin by the cylinder-plate method (3) we have been led to test the effect of trace amounts of cobalt on penicillin activity (4), and we have observed that the effectiveness of penicillin can be markedly increased by proper use of suitable concentrations of cobalt. Addition of trace amounts of cobalt to the nutrient agar in test plates increases approximately four- to eightfold the effectiveness of relatively dilute penicillin solutions in producing inhibition zones on plates seeded with *Staphylococcus aureus* and decreases to approximately one-third or less, depending upon the test organism that is used, the minimum or threshold concentration of penicillin that can be detected by the cylinder-plate technic.

EXPERIMENTAL

Results of a typical experiment in which different amounts of cobalt chloride were incorporated in the test agar are plotted in Fig. 1A. Ordinates represent zone diameters corresponding to the different concentrations of penicillin plotted on the abscissae. The data are from plates seeded with *Staphylococcus aureus* NRRL 313 (same as FDA strain 209P) and processed by the three-hour technic described earlier (3). The penicillin used throughout this work was a crystalline preparation of sodium penicillin G that assayed 1560 u./mg. The concentration of 1 mg. $CoCl_2 \cdot 6H_2O$ per L. has been most effective for inducing the enhancing action in our experiments,

* Received Oct. 16, 1947, from the University of California College of Pharmacy, The Medical Center, San Francisco, Calif.

† Acknowledgment is made to the Cutter Laboratories, Berkeley, Calif., for a generous grant that supported a portion of this research.

Antibacterial Activity of Combinations of Iodine and Human Blood Fractions*,†

By LOUIS GERSHENFELD and BERNARD WITLIN

Iodine was combined with whole citrated human blood and with several fractions of this material. Physical and bacteriostatic properties of these iodine compounds were determined and the results are reported.

GERSHENFELD and Miller (1) previously reported that mixtures of whole blood with iodine solutions resulted in the taking up of the free iodine by the blood and under certain conditions there was a retention of antibacterial efficiency by the blood-iodine mixture.

Iodinated horse serum has been used as a chemotherapeutic agent in various bacterial diseases. Very little information as to the exact method of preparation and to its therapeutic value is available. One marketed preparation, Iodalbin,[1] recommended for parenteral use is reported as containing 21.5 per cent iodine combined with the horse serum proteins. Muus, Coons, and Salter (2) prepared iodinated horse serum albumin and reported that the amount of iodine which entered into the protein molecule increased with the addition of iodine up to the maximum, which they reported to be 15 per cent. The exact changes produced in various proteids by iodination have not been determined but distinct differences have been observed.

This study has been undertaken in an attempt to note if the various human blood-iodine combinations possess bacteriostatic or bactericidal efficiencies and to determine if possible the blood fractions which combine to form such bacteriostatic or bactericidal compounds.

EXPERIMENTAL

The following whole human blood and fractions thereof were employed: citrated whole blood; erythrocytes washed free of citrated plasma; dried human blood cells[2]; citrated normal human plasma[3] (65 Gm. protein/L.); fibrinogen[3] (3 Gm. protein/L); gamma and beta globulins[3] (20 Gm. protein/L.); alpha globulin and lipids[3] (5 Gm. protein/L.); alpha and beta globulins[3] (4.5 Gm. protein/L.); albumin[3] (29 Gm. protein/L); and albumin and alpha globulin[3] (1 Gm. protein/L.).

Test for Bactericidal Efficiency of Iodine-Blood Protein Mixtures.—To 10-cc. volumes of each of the above solutions, suspensions or powders, a 5% aqueous iodine-potassium iodide solution (Lugol's) was added in varying amounts at 22.5°.

Bactericidal efficiency tests were performed in accordance with the Food and Drug Administration technic (3) employing *Staphylococcus aureus* at 37°.

The iodine-blood and iodine-blood fraction mixtures were tested for bactericidal efficiency immediately after preparation and also on the first, second, third, fourth, and fifth day thereafter.

Findings.—In all instances, iodine-treated citrated human blood, hemoglobin, plasma and its fractions failed to reveal bactericidal efficiencies other than those which could be attributed to the presence of free iodine.

Absorption of Iodine by Blood and Its Derivatives at Varying Temperatures and the Testing of the Resulting Products.—To 250 cc. portions of human citrated blood and of the various fractions already described, there were added 250 cc. distilled water and 18 cc. of 50% aqueous iodine solution (10 X Lugol's). One series of each was prepared at each of the following temperatures: 22.5°, 37° and 56°.

The precipitates formed were washed with distilled water until negative for free iodine. They were dried at room temperature and tested for bacteriostatic efficiency by the F.D.A. Agar-Plate technic employing *S. aureus* at 37°. The results are reported in Table I.

Findings.—(*a*) Larger amounts of iodine went into combination with blood and its derivatives at 56° than at 37° and more at 37° than at 22.5°.

(*b*) Washed precipitates of blood-iodine mixtures prepared at 56° possessed greater bacteriostatic properties (as revealed by inhibition zones) than those prepared at 37° or 22.5°.

(*c*) Precipitates prepared by the combination of iodine with albumin fractions, plasma or with egg albumin and washed until all the free iodine had been removed did not display bacteriostatic proper-

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[1] "Iodalbin," Parke, Davis & Company.

[2] "Lyocyte" Powder, Sharp & Dohme, Inc.

[3] Supplied through the courtesy of the Department of Physical Chemistry, Harvard Medical School, and prepared under contract between the Office of Scientific Research and Development and Harvard University.

ties (as revealed by the nonproduction of inhibition zones).

(*d*) Larger inhibition zones were obtained with iodine-alpha and beta globulin mixtures than with alpha globulin and lipids or gamma and beta globulins or any of the other fractions.

Bacteriostatic Efficiency of Iodine-Plasma Compounds Prepared by the Gradual Addition of 10 X Strength Lugol's Iodine Solution to Aqueous Dilutions of Human Blood Plasma and Its Fractions (see Table II).—(*a*) To 250 cc. citrated human blood plasma there were added 250 cc. distilled water in a 1000-cc. beaker. The latter, containing the diluted plasma, was kept in a water bath at the desired temperature and constantly stirred with an electrically operated stirring apparatus. Ten X strength Lugol's iodine solution was added in 0.1-cc. amounts until free iodine was no longer present. The mixture was filtered and the precipitate was washed with distilled water until negative for free iodine (see Table II). Precipitates were then examined for bacteriostatic efficiency by the F.D.A. Agar-Plate technic against *S. aureus* at 37°.

TABLE I.—BACTERIOSTATIC EFFICIENCY OF IODINATED HUMAN BLOOD, PLASMA AND PLASMA FRACTIONS AT VARYING TEMPERATURES

Substance	Mixture made at Temp. ° C.	Washed Precipitate (Iodine-Free) F.D.A. Agar-Plate (*S. aureus*) Zone in Mm.
Whole blood (citrated)	22.5	12
	37.0	15
	56.0	17
Hemoglobin	22.5	12
	37.0	15
	56.0	17
Plasma (citrated)	22.5	5
	37.0	8
	56.0	10
Fibrinogen	22.5	5
	37.0	5
	56.0	8
Gamma and beta globulins	22.5	5
	37.0	6
	56.0	8
Alpha and beta globulins	22.5	15
	37.0	17
	56.0	20
Albumin	22.5	0
	37.0	0
	56.0	0
Albumin and alpha globulin	22.5	0
	37.0	0
	56.0	2
Alpha globulin and lipids	22.5	5
	37.0	7
	56.0	8
Fibrin foam[a]	22.5	0
	37.0	0
	56.0	0
Controls		
Iodalbin	..	10
Egg albumin	22.5	0
Egg albumin	37	0
Egg albumin	56	0
2% aqueous iodine	...	10 (cup plate)
5% aqueous iodine	...	10 (cup plate)
2% iodine tincture (U. S. P.)	...	16 (cup plate)
7% strong iodine tincture (N. F.)	...	16 (cup plate)

[a] Fibrin foam, Sharp & Dohme, Inc.

TABLE II.—AGAR-PLATE TEST. INHIBITION ZONES OBTAINED WITH IODINE-HUMAN BLOOD PLASMA COMPOUNDS PREPARED AT VARYING TEMPERATURES

Compound Prepared Using 250 Cc. Plasma and 250 Cc. Distilled Water with 10 X Lugol's Solution at Temp., ° C.	10 X Lugol's Used, Cc.	Zone of Inhibition (*S. aureus*), Mm.
22.5	1.5	1.0
37	6.0	2.5
45	12.0	5.0
56	21.5	10.0

Findings.—The amount of iodine contained in the compounds was dependent upon the temperature at which combination took place. The iodine content was greater in the product prepared at 56° than at 45°, greater at 45° than at 37°, and greater at 37° than at 22.5°.

These iodine-plasma combinations produced brown loose powders, insoluble in water, alcohol, or acid, but soluble in fixed alkalies, ammonia, and alkali carbonates. They were precipitated from their solutions by acids but went into solution with an excess of acid.

Iodine-plasma mixtures formed by the interaction between iodine and human blood plasma gave inhibition zones on F.D.A. Agar-Plate tests, the diameter of which varied depending upon the temperature which prevailed at the time of combination (or preparation).

Little is known as to the exact changes produced in proteids by iodination, but distinct differences are apparent, depending upon the temperature used and the time permitted for the iodination. The heating of a proteid solution produced a marked change upon the proteins present. Each increase in temperature affected a different proteid or group of proteids until a maximum coagulation or precipitation of proteids occurred, each responding differently to iodination.

Bacteriostatic Efficiency of Precipitates Formed by Iodine-Plasma Mixture at 22.5° and Washed Until All the Free Iodine Was Removed.—Individual precipitates were washed separately with either distilled water, 95% alcohol, benzin, carbon tetrachloride, iso-amyl alcohol, ethyl dichloride, or acetone. Individual samples of the precipitates were tested by the F.D.A. Agar-Plate technic in the wet and dry state. Samples of each of the precipitates were dried at 22.5° and 37.5°. A portion of each precipitate was then moistened with some of the solvent originally used in its preparation. The

TABLE III.—INHIBITION ZONES OBTAINED WITH IODINE-HUMAN BLOOD PLASMA PREPARED AT 22.5° AND WASHED WITH DIFFERENT SOLVENTS (*S. aureus*)

Lugol's Solution, Cc.	Plasma, Cc.	Solvent	Wet Zone, Mm.	Dried 22.5° Zone, Mm.	Dried 37° Zone, Mm.	Re-moistened Zone, Mm.
2.0	10	Distilled water	4	2	2	2
2.0	10	95% alcohol	5	0	0	0
2.0	10	Benzin	3	0	0	0
2.0	10	Carbon tetrachloride	1	0	0	0
2.0	10	Ethyl dichloride	2	0	0	0
2.0	10	Acetone	10	0	0	0
Controls		Distilled water	0	Cup plate	..	..
Controls		95% alcohol	0	Cup plate	..	..
Controls		Benzin	0	Cup plate	..	..
Controls		Carbon tetrachloride	0	Cup plate	..	..
Controls		Iso-amyl alcohol	0	Cup plate	..	..
Controls		Ethyl dichloride	0	Cup plate	..	..
Controls		Acetone	0	Cup plate	..	..

re-moistened precipitates were tested by the F.D.A. Agar-Plate technic with *S. aureus* at 37°.

Findings.—Iodine plasma precipitates prepared in distilled water at 22.5° and washed with distilled water until all the free iodine was removed displayed bacteriostatic activity when tested by the F.D.A. Agar-Plate technic. Precipitates were washed once with distilled water or the organic solvents and permitted to dry. They were then re-washed repeatedly with distilled water or the solvents until free iodine was removed. After drying, the precipitates were found to retain quantities of iodine comparable to those prepared at identical temperatures (Table II).

Iodine plasma precipitates prepared at 22.5°, washed with organic solvents, dried at 22.5° and 37° and subsequently employed in the above test did not display bacteriostatic efficiency. When the water-washed precipitates were dried at 22.5° or 37°, re-moistened with distilled water, and tested by the F.D.A. Agar-Plate technic, varying degrees of bacteriostasis were displayed as indicated by the zones of inhibition (see Table III).

SUMMARY AND CONCLUSIONS

1. Iodine combined with whole citrated human blood, hemoglobin, citrated plasma, fibrinogen, gamma and beta globulins, alpha globulin and lipids, alpha and beta globulins, and albumin and alpha globulin to form apparently stable water insoluble complex protein compounds. The iodine in combination in these compounds was readily removed by organic solvents.

2. Iodine combined with the albumin fraction of plasma and with egg albumin to form precipitates. These precipitates did not exhibit bacteriostatic or bactericidal activity after all free iodine had been removed by washing with distilled water or organic solvents

3. Larger amounts of iodine went into combination with blood and its derivatives at 56° than at 37° and more at 37° than at 22.5°.

4. Precipitates of whole citrated blood-iodine and plasma-iodine mixtures prepared at 56° and washed free of free iodine with distilled water possessed greater bacteriostatic properties than those prepared at 37° or 22.5°.

5. Precipitates of human blood plasma-iodine mixtures, filtered and permitted to dry, did not relinquish the bound iodine when repeatedly washed again with distilled water or organic solvents. Precipitates of human blood plasma-iodine mixtures repeatedly washed, immediately after their preparation, with distilled water or organic solvents readily liberated the iodine from the precipitate into the solute.

6. Greater bacteriostatic properties were obtained with iodine-alpha and beta globulin mixtures than with iodine-gamma and beta globulin mixtures or iodine-alpha globulin and lipid mixtures.

REFERENCES

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(2) Muus, J., Coons, A. H., and Salter, W. J., *J. Biol. Chem.*, 139, 135(1941).
(3) *U. S. Dept. Agr., Food, Drug Admin., Tech. Bull. No.* 198, 1931.

A Quantitative and Qualitative Evaluation of Official and Unofficial Species of Psyllium Seeds and Their Mucilages*

By DAVID GREENBERG†,‡,§

A convenient method for the quantitative determination of mucilage in Psyllium seed is described. Two native unofficial Plantago seeds are described and compared with those now official.

THE following study of psyllium seeds and their respective mucilages was initiated when a sample of *Plantago wrightiana* Decne was presented to the author for comparison with the commercial psyllium seeds. The large size of the seed, which yielded a copious amount of bland-tasting mucilage, and the hardiness and high seed yield of the wild plant indicated a possible native source of psyllium seeds. Subsequent swelling tests indicated a very high percentage of mucilage. The war-born shortages of imported psyllium seed and the present national emphasis placed on providing, when possible, an economically feasible native supply of crude drugs led to a more critical investigation of *P. wrightiana* seeds in comparison with the imported psyllium seeds.

The present study of the various psyllium seeds and their mucilages was undertaken: (*a*) to provide evidence that the seeds of *P. wrightiana* are a possible source of commercial plantago seeds; (*b*) to establish a convenient and practicable means of determining the percentage content of available mucilage[1] in psyllium seeds; (*c*) to suggest a method whereby the available mucilage of psyllium seeds may be obtained in a pure form for commercial or experimental use; (*d*) to question the long-standing view that the swelling factor of psyllium seeds is indicative of the mucilage content, and to suggest that it is instead dependent on the physical properties of the mucilage; (*e*) to present some of the physical properties of mucilage solutions as a means of evaluating these products; (*f*) to determine if, within the same species of seed, the quantity of mucilage, the swelling factor, and the viscosity of definite dilutions of the pure mucilage differ significantly.

The evaluation of psyllium seeds is at present based on the National Formulary VIII *Test for Quality* in the monograph "Plantago Seed" (1). Important and more exacting test standards, as modifications of the official method, have been devised by Youngken (2). These improvements take into consideration the importance of temperature, diameter of the graduated cylinder, and a means of agitating the seeds during the test period. It will be noted, however, that these factors considered by Youngken relate to a fundamentally important property of the seed mucilage which in the final analysis determines the swelling factor. This property, the viscosity of the hydrated mucilage formed, when improperly interpreted, is undoubtedly the underlying cause for many of the discrepancies arising during swelling factor determinations made by different investigators. Clevenger (3), in one of the first attempts to evaluate psyllium seed qualitatively, used a 10-cc. cylinder and 1 Gm. of seed. The swelling factors obtained were low because of the impaction of the seed-mucilage mass within the small tube. Burlage (4), in a report on a collaborative study of the swelling factors of psyllium seeds based on the improved Youngken method, presents data which for the most part are consistent, but nevertheless display some wide discrepancies. If, as stated by Burlage, "The swelling factor is indicative of the mucilage content..." (4), such wide variation of swelling factors as 4.5 cc. within the same species is in need of explanation and, if possible, correction.

Aside from the excellence and importance of the contributions to the chemistry of the mucilages, it should be noted that the low percentages of mucilages obtained for chemical analysis by Nelson and Percival (5) and Mullen and Percival (6) leave considerable doubt as to the applicability of the information so obtained to the chemistry of the total mucilage. The 5% of mucilage obtained from the seeds studied in each case obviously represents only a portion of the available mucilage from these seeds.

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† Recipient of Kilmer Prize for 1947.
‡ Now instructor in pharmacognosy, University of Texas.
§ Acknowledgment is made to Dr. C. C. Albers, Professor of Pharmacognosy, University of Texas, for his assistance.
[1] The term "available mucilage" is used here to denote that mucilage which takes a direct part in the swelling factor and which can be obtained from the seed free of underlying seed parts.

Fig. 1.—*Plantago arenaria* No. 1 and No. 2. This photograph is intended to show that these two samples are almost indistinguishable as to color, size, and shape.

Fig. 2.—*Plantago rhodosperma*. The seeds of *P. rhodosperma* are broadly ellipsoidal, pear-shaped, 2.5–3 mm. long and 1.5 mm. wide, light to dark red in color. The dorsal surface exhibits a large lighter-colored embryo broad at the small end of the seed and tapering off to a point at the edge of the broad end of the seed. The seed is bordered by a thin, translucent band. The ventral surface is flat to slightly convex. The edge is bordered by a clear band blending into the red center in which lies the conspicuous, white hilum. The two surfaces are dull and slightly reticulated.

The author assumes that the chemical investigation of seed mucilages should be made on the total mucilage from that seed or that cognizance be taken of the fact that the information obtained from the study of fractions thereof may not represent the true chemical picture. This assumption is given factual support when consideration is taken of the observations of Anderson and Fireman (7) who detected significant variations in the chemical composition of mucilage obtained from the same lot of seed with slight variations in the manner of preparing the pure mucilage. Variations in the amount of water and pressure as well as the time factor used to extract the mucilage yielded differences in the chemical constitution of the final product.

While this author is in complete agreement with Anderson, Gillette, and Seeley (8) in their recognition of the fact that a knowledge of the composition and structure of the mucilages is important as a stepping stone in the study of the more complex cell wall constituents, he again suggests that it is of importance also that the mucilage studied represent as nearly as possible the total mucilage layer, free of underlying seed parts.

MATERIALS AND APPARATUS[2]

The seeds designated in this study as *Plantago ovata* Forskal No. 1, *P. arenaria* Waldstein et Kitaibel No. 1 and No. 2 (Fig. 1) were obtained from a commercial dealer in crude drugs. *P. ovata* No. 2, *P. arenaria* No. 3, and *P. arenaria* No. 4[3] were obtained from a second commercial source. The seeds of *P. rhodosperma* Decne, identified by Dr. B. C. Tharp, Curator, Herbarium, The University of Texas (Fig. 2) were collected by hand in late June,

Fig. 3.—Herbarium specimen of mature plant of *Plantago rhodosperma*.

[2] Appreciation is also expressed to Mr. C. L. York, graduate student in Botany, The University of Texas, for his assistance in preparing all photographs.

[3] This box of seed was plainly labeled *Plantago psyllium seed*, but because of the size and shape of the seed, their swelling factors and mucilage content, and the color of the hydrated mucilage, these seeds will be designated as *P. arenaria* No 4 in this study.

Fig. 4.—*Plantago wrightiana.* The seeds of *P. wrightiana* are elongated-ellipsoidal, boat-shaped, from 3–4 mm. in length and 1.5–2 mm. wide, pale brown with a dull reticulated surface, slightly darker brown along the margin, ending in a transparent edge. The convex dorsal surface exhibits a distinct transverse groove at the widest portion of the seed which bisects the lighter colored embryo area. The concave ventral portion is bordered by a 0.5-mm. ridge sloping into a deep concavity in the center of which lies the dark hilum surrounded by a conspicuous white border.

1946, in Austin, Tex., from plants which had been mowed down and left on the edge of a former hay field. These seeds as a whole probably do not represent the best obtainable from this plant. Figure 3 is a photograph of a herbarium sheet of *P. rhodosperma.* This plant is distributed throughout the sandy soil of Missouri and Oklahoma to Louisiana, Texas, and Arizona.

The *P. wrightiana* seeds (Fig. 4), also identified by Dr. B. C. Tharp, were obtained from plants grown in the Medicinal Plant Garden of The College of Pharmacy, The University of Texas, during 1945 and 1946. *P. wrightiana,* under cultivation, grows well on marginal and submarginal soil, favoring a deep sand, requiring little attention and no irrigation. The plant is native to Texas and Arizona and is found in dry sandy soil. When planted in October or November the mature seeds may be harvested in May or June. The fruiting spikes extend erect over the tops of the leaves with sufficient clearance for easy harvesting with a modified combine. Figure 5 is a photograph of a mature plant of *P. wrightiana* mounted in The University of Texas Herbarium.

Attempts were made to obtain samples of *P. psyllium* L., in order to complete this study on the official psyllium seeds, but none was available from the sources contacted.

Work is contemplated on the following plantago seeds native to Texas when material becomes available: *P. occidentalis* Decne, *P. helleri* Small, *P. spinulosa* Decne, *P. aristata* Michx., *P. virginica* L., and *P. inflexa* Morris.

The hand press (Fig. 6) used in the quantitative determinations reported in this study was designed by the author and tooled in the University Machine Shop. The interchangeable sieve plate allows for

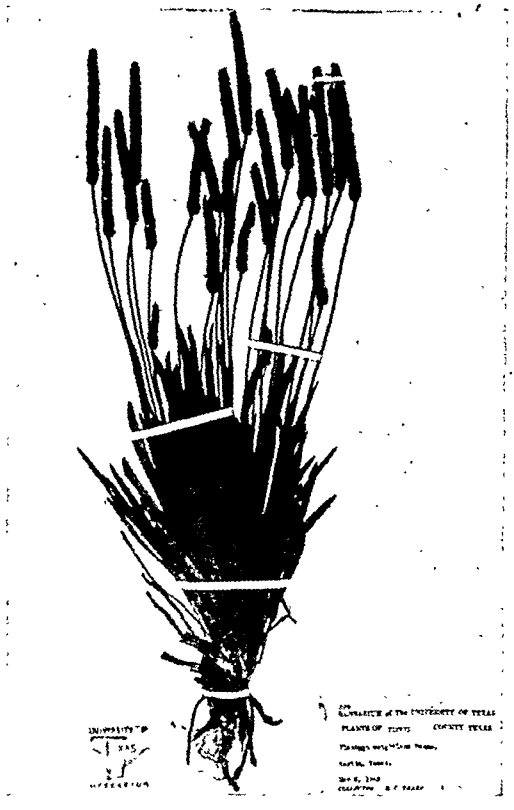

Fig. 5.—Herbarium specimen of mature plant of *Plantago wrightiana.*

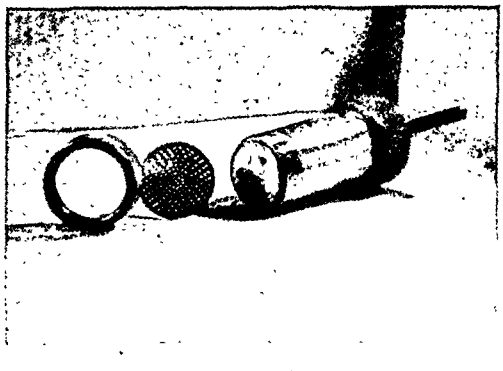

Fig. 6.—Hand Press. The hand press, except for the sieve plate, is made of stainless steel. The sieve plate is brass. The essential dimensions are as follows: The cylinder is $3^7/_8$-inches long by $1^5/_8$-inches inside diameter; thread on the $^1/_2$-inch push rod connecting the 5-inch handle to the piston are $^1/_2$-inch N.C. thread. The sieve plate is $^1/_8$-inch by $1^{53}/_{64}$-inches with approximately 200 No. 57 drill holes. The push rod is connected to the piston by a ball and sprocket joint. The piston is covered with a leather gasket press-fitted to the cylinder and screwed to the piston. The bottom ring is flanged to hold the sieve plate in place as the ring is screwed onto the cylinder.

the use of plates with holes of different sizes when smaller or larger seeds than those in this study are used. The hand press should be constructed entirely of a rust-resistant metal, monel or stainless steels. If a large size hand press is desired for the expression of larger amounts of mucilage, the diameter of the cylinder should be increased proportionately to avoid the occlusion of the sieve plate holes by the pressure of a long column of seed-mucilage mass on the mucilage-free seeds nearest the plate.

EXPERIMENTAL

Swelling Factors.—In attempts to find a satisfactory explanation for the wide variations in the swelling factors of a given lot of seeds obtained by Burlage (4) and others, the following three distinct methods of procedure were followed. The results of these determinations are presented in Table I.

TABLE I[a]

Seed	Minimum N. F. Swelling Factor, Cc.	Average Swelling Factors, Cc. Method 1	Method 2	Method 3	Percentage of Mucilage 20 Cc. H_2O	30 Cc. H_2O	Density	Absolute Viscosity in Centistokes
P. arenaria No. 1	8.0	7.0	7.0	7.0	9.00	8.16	1.0024	5.87
P. arenaria No. 2	8.0	9.5	8.0	17.0	7.66	7.36	0.9999	96.00
P. arenaria No. 3	8 0	18.0	7.0	10.0	6.95	....	1.0023	4.32
P. arenaria No. 4	8.0	18.0	6.0	14.0	8.93	...	1.0026	2.68
P. ovata No. 1	10.0	11 0	9.5	11.0	22.30	21.70	1.0023	19.38
P. ovata No. 2	10.0	13.0	10.0	13.0	20.50	....	1.0024	12.00
P. wrightiana	...	18.0	14.5	20.0	20.50	23.00	0.9934	518.40
P. rhodosperma	...	19.0	...	...	16.00[b]	15.11[b]	0.9970	Too thick

a All figures in this table represent averages of at least duplicate determinations.
b Further soaking and expression of mucilage yielded 18–20%. The high viscosity of this mucilage does not permit of complete expression under these conditions.

From these figures it is evident that the possible swelling factors obtainable from a given lot of seeds run from one extreme to another, with none having much meaning unless the method used is rigidly standardized.

Method No. 1.—Tests on 1-Gm. samples of seed were carried out as directed by the National Formulary VIII, page 336, with the interpretation of the phrase, "Shake the cylinder at intervals during 24 hours," as apparently previous workers have done. The seeds were placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder was immediately inverted several times to disperse the seeds and allowed to stand for *several hours* after which time the cylinder was similarly shaken at several-hour intervals for twenty-four hours.

Method No. 2.—One gram of seed was placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder is inverted *once* to disperse the seeds and the cylinder allowed to remain undisturbed for twenty-four hours, after which the reading is taken.

Method No. 3.—One gram of seed was placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder is then shaken constantly and vigorously by complete inversion of the cylinder for the first hour and allowed to settle for the remaining twenty-three hours, after which the reading is taken.

It is understandable that the wholesale dealer in *P. arenaria* No. 4 (labeled as *P. psyllium*), by disregarding the macroscopic features of the seed, and without more exacting test standards, might easily interpret the high swelling factor as meeting the National Formulary minimum of 14 cc. for *P. psyllium*.

Similar differences in the results of early quantitative determinations on the same lot of seed were obtained by the author until the factor of adequate and correctly timed agitation was considered.

Quantitative Determination of Mucilage Content.—The current usage of psyllium seed mucilages, in their purified state, in medicine and industry requires a simple means of determining the quantity of mucilage available from a given lot of seed. This information is of value to the importer of the seed, and to the manufacturer of the mucilage product. The data obtainable from the pure mucilage, if they represent the total mucilage, are of value to the chemist, the doctor who prescribes the product as a laxative, and the manufacturer of cosmetics and other mucilage-containing preparations.

The proper use of the above-described hand press, which can be easily and inexpensively made in any well-equipped machine shop, will accomplish these results consistently.

The author has found that the proportion of 1 Gm. of seed per 20 cc. of water soaked for twenty hours with considerable agitation during the first hour and occasional shaking thereafter produces results which are very constant for each lot of seed and as a whole is suitable as a standard for routine determinations. The use of 30 cc. of water for each 1 Gm. of seed gave results (see Table I) which were lower in some instances and higher in others than when 20 cc. was used.

The following method was used in the determinations reported in Table I: 1.5 Gm. of seed was

macerated with 30 cc. of distilled water for twenty hours with vigorous agitation during the first hour to insure the dispersion of the mucilage formed and to break up the agglutinated clumps of seed which formed in most cases immediately upon the addition of water to the seeds. The material was then poured into the hand press, the sieve plate put in place, and the mucilage expelled by turning the handle rapidly. The thread ratio on the push rod is such as to allow rapid turning and maximum pressure with the least exertion. The adhering mucilage is removed from the outside of the sieve plate with a spatula and added to the bulk collected on a glass plate or in a tared Petri dish bottom. The mucilage is then dried in an oven or on a temperature-controlled hot plate not above 100°. The author used a steel plate 3 feet by 4 feet by $^1/_8$ inch on the underside of which was soldered 50 feet of copper tubing carrying steam. This allowed rapid drying at 95–100°. When dry the mucilage may be shaved from the glass with a single-edge razor blade and weighed, or if in a tared dish the weight may be determined directly.

As may be seen from the above description of method and the data presented in Table I, the quantitative determination of mucilage content can be easily and consistently determined. The percentages averaged and presented in Table I for each lot of seed varied for the most part within 1%.

It should be noted here that in expressing the mucilage from the seeds as described above, the seeds are left free of any mucilaginous feeling and are not crushed. A few seeds are occasionally forced through the sieve plate holes but may be easily removed when the mucilage is dry.

The question as to the completeness with which the mucilage is removed by this process was solved by re-soaking the seeds in fresh water, after the single expression, and again expelling the water. When dried this last volume of wash water yielded only a negligible amount of mucilage which can be accounted for as that small amount of mucilage remaining compressed between the seeds after the first expression. This further soaking also softens the seed and permits crushing.

Density of Mucilage Dilutions.—The determination of the density of the mucilage solutions used in determining the viscosity was necessary for computing the absolute viscosity. The data obtained are presented here because of their interesting variations. Further work is contemplated on this feature. For these determinations a dilution of 150 mg. of mucilage per 20 cc. of water was made and carried out at 28° using a 25-cc. pycnometer. A comparative study of the osmotic pressure values of the various mucilages is also contemplated.

Viscosity.—It is the viscosity values presented in Table I which show the great differences between mucilages obtained from different species of Plantago seeds as well as the vast difference between mucilages obtained from two lots of seed of the same species. These determinations were made with a Ubbelohde viscosimeter at 37.8° with dilutions of mucilage containing 150 mg. per 20 cc. of distilled water. The viscosimeter used was calibrated for water at 37.8° and the values obtained on the mucilages were correspondingly corrected.

The dried mucilage was added to the water in a test tube and heated to 100° for one to five minutes to facilitate the hydration of the mucilage, and then cooled to 37.8°. The mucilage was introduced into the viscosimeter which rested in a water bath. Determinations were made in rapid succession and an average taken. After four or five determinations, the mucilage drawn into the upper bulbs of the viscosimeter became successively less viscous, with the exception of that of the four specimens of *P. arenaria*. This is readily explained in the case of the mucilage of *P. ovata* which separates fairly rapidly, leaving a watery supernatant liquid occupying as much as one-half the volume in the test tube in a period of several days when undisturbed. The tubes of mucilage of *P. wrightiana*, *P. rhodosperma*, and *P. arenaria* No. 1 and No. 2 showed no signs of separation after one week, while those of *P. arenaria* No. 3 and No. 4 and *P. ovata* No. 1 and No. 2 separated within twenty-four hours. It is possible that the drawing of the suspension through the capillary tube causes a separation more rapidly than when standing undisturbed. Caution should be observed to avoid the introduction of bubbles into the viscous suspensions as these bubbles reduce the viscosity far out of proportion to the volume they occupy. Bubbles may be removed from the more viscous mucilage by gentle warming.

The viscosity values of seed mucilages should prove of considerable importance in evaluating the mucilage or the seed. To the manufacturer of liquid cosmetic preparations or of dry preparations intended to be wetted in their use, this factor is of utmost importance.

Description of Dry Mucilage.—The dried pure mucilage from each of the lots of seeds used in this study varied in texture and color. The mucilages of the four lots of *P. arenaria* each differ significantly from the others in color. Upon scraping the dried mucilage from the glass plate, that of *P. arenaria* No. 1 shatters into a very fine spray of glistening particles which are carried off by slight currents of air, while that of *P. arenaria* No. 2, No. 3, and No. 4 fragment without shattering or tending to be thrown into the air. The mucilages of *P. arenaria* No. 1 and No. 2 differ only slightly in color, both being a dark reddish brown with some luster. *P. arenaria* No.3 mucilage is a faint gray brown with considerable luster. That of *P. arenaria* No. 4 is a medium brown with some luster. *P. ovata* No. 1 and No. 2 mucilages are similar, both being easily removed from the glass plate in short curled strips. These strips are transparent but en masse appear to be a dull white. The mucilages of *P. rhodosperma* and *P. wrightiana* are similar to that of *P. ovata* but tend to break up into shorter strips. In all the above species the thin layer of mucilage on the plate is clear and transpar-

the use of plates with holes of different sizes when smaller or larger seeds than those in this study are used. The hand press should be constructed entirely of a rust-resistant metal, monel or stainless steels. If a large size hand press is desired for the expression of larger amounts of mucilage, the diameter of the cylinder should be increased proportionately to avoid the occlusion of the sieve plate holes by the pressure of a long column of seed-mucilage mass on the mucilage-free seeds nearest the plate.

EXPERIMENTAL

Swelling Factors.—In attempts to find a satisfactory explanation for the wide variations in the swelling factors of a given lot of seeds obtained by Burlage (4) and others, the following three distinct methods of procedure were followed. The results of these determinations are presented in Table I.

TABLE I[a]

Seed	Minimum N F Swelling Factor, Cc.	Average Swelling Factors, Cc. Method 1	Method 2	Method 3	Percentage of Mucilage 20 Cc. H_2O	30 Cc. H_2O	Density	Absolute Viscosity in Centistokes
P. arenaria No. 1	8.0	7.0	7.0	7.0	9.00	8.16	1.0024	5.87
P. arenaria No. 2	8.0	9.5	8.0	17.0	7.66	7.36	0.9999	96.00
P. arenaria No. 3	8.0	18.0	7.0	10.0	6.95		1.0023	4.32
P. arenaria No. 4	8.0	18.0	6.0	14.0	8.93		1.0026	2.68
P. ovata No. 1	10.0	11.0	9.5	11.0	22.30	21.70	1.0023	19.38
P. ovata No. 2	10.0	13.0	10.0	13.0	20.50		1.0024	12.00
P. wrightiana	. .	18.0	14.5	20.0	20.50	23.00	0.9934	518.40
P. rhodosperma	. . .	19.0	. . .	. . .	16.00[b]	15.11[b]	0.9970	Too thick

a All figures in this table represent averages of at least duplicate determinations.
b Further soaking and expression of mucilage yielded 18–20%. The high viscosity of this mucilage does not permit of complete expression under these conditions

From these figures it is evident that the possible swelling factors obtainable from a given lot of seeds run from one extreme to another, with none having much meaning unless the method used is rigidly standardized.

Method No. 1.—Tests on 1-Gm. samples of seed were carried out as directed by the National Formulary VIII, page 336, with the interpretation of the phrase, "Shake the cylinder at intervals during 24 hours," as apparently previous workers have done. The seeds were placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder was immediately inverted several times to disperse the seeds and allowed to stand for *several hours* after which time the cylinder was similarly shaken at several-hour intervals for twenty-four hours.

Method No. 2.—One gram of seed was placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder is inverted *once* to disperse the seeds and the cylinder allowed to remain undisturbed for twenty-four hours, after which the reading is taken.

Method No. 3.—One gram of seed was placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder is then shaken constantly and vigorously by complete inversion of the cylinder for the first hour and allowed to settle for the remaining twenty-three hours, after which the reading is taken.

It is understandable that the wholesale dealer in *P. arenaria* No. 4 (labeled as *P. psyllium*), by disregarding the macroscopic features of the seed, and without more exacting test standards, might easily interpret the high swelling factor as meeting the National Formulary minimum of 14 cc. for *P. psyllium.*

Similar differences in the results of early quantitative determinations on the same lot of seed were obtained by the author until the factor of adequate and correctly timed agitation was considered.

Quantitative Determination of Mucilage Content.—The current usage of psyllium seed mucilages, in their purified state, in medicine and industry requires a simple means of determining the quantity of mucilage available from a given lot of seed. This information is of value to the importer of the seed, and to the manufacturer of the mucilage product. The data obtainable from the pure mucilage, if they represent the total mucilage, are of value to the chemist, the doctor who prescribes the product as a laxative, and the manufacturer of cosmetics and other mucilage-containing preparations.

The proper use of the above-described hand press, which can be easily and inexpensively made in any well-equipped machine shop, will accomplish these results consistently.

The author has found that the proportion of 1 Gm. of seed per 20 cc. of water soaked for twenty hours with considerable agitation during the first hour and occasional shaking thereafter produces results which are very constant for each lot of seed and as a whole is suitable as a standard for routine determinations. The use of 30 cc. of water for each 1 Gm. of seed gave results (see Table I) which were lower in some instances and higher in others than when 20 cc. was used.

The following method was used in the determinations reported in Table I: 1.5 Gm. of seed was

macerated with 30 cc. of distilled water for twenty hours with vigorous agitation during the first hour to insure the dispersion of the mucilage formed and to break up the agglutinated clumps of seed which formed in most cases immediately upon the addition of water to the seeds. The material was then poured into the hand press, the sieve plate put in place, and the mucilage expelled by turning the handle rapidly. The thread ratio on the push rod is such as to allow rapid turning and maximum pressure with the least exertion. The adhering mucilage is removed from the outside of the sieve plate with a spatula and added to the bulk collected on a glass plate or in a tared Petri dish bottom. The mucilage is then dried in an oven or on a temperature-controlled hot plate not above 100°. The author used a steel plate 3 feet by 4 feet by $^1/_8$ inch on the underside of which was soldered 50 feet of copper tubing carrying steam. This allowed rapid drying at 95–100°. When dry the mucilage may be shaved from the glass with a single-edge razor blade and weighed, or if in a tared dish the weight may be determined directly.

As may be seen from the above description of method and the data presented in Table I, the quantitative determination of mucilage content can be easily and consistently determined. The percentages averaged and presented in Table I for each lot of seed varied for the most part within 1%.

It should be noted here that in expressing the mucilage from the seeds as described above, the seeds are left free of any mucilaginous feeling and are not crushed. A few seeds are occasionally forced through the sieve plate holes but may be easily removed when the mucilage is dry.

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ent with *P. wrightiana* exhibiting denser splotches spread throughout the clear matrix. The splotches are observed to be what is apparently incompletely hydrated mucilage forcibly expressed from the seed and retaining, in a swollen, jelly-like mass, the concavity of the mucilage layer around the dorsal side of the seed.

Description of the Mucilage Dilutions.—A visual inspection of the tubes containing the dilutions of mucilage used in the viscosity and density determinations disclosed many differences. In all cases the mucilage formed is a clear, transparent liquid. The dilutions of *P. arenaria* No. 1 and No. 2 differ slightly in color, both being a light brown with a suggestion of red. *P. arenaria* No. 3 is a much lighter, dirty brown color. *P. arenaria* No. 4 is medium brown in color. The mucilages of *P. arenaria* No. 1, No. 3, and No. 4 are thin liquids allowing bubbles of air introduced when shaken vigorously to rise rapidly to the top. That of *P. arenaria* No. 2, *P. rhodosperma*, and *P. wrightiana* exhibit a ropy consistency when the tube is rotated on a horizontal plane and will retain large bubbles of air over a period of several days. The mucilage dilutions of the two samples of *P. ovata* are free flowing and do not retain bubbles of air introduced into them by shaking. The 150 mg. per 20-cc. dilution of *P. rhodosperma* yields a semisolid, nonflowing mass, very closely approaching the consistency of "set" gelatin. This was too viscous to use in the Ubbelohde viscosimeter.

DISCUSSION

The author has observed that the factors involved in the National Formulary *Test for Quality* do not comprise a simple relationship. It can readily be seen that the variables involved in the swelling factor determination, mainly the amount of agitation of the seeds in water, the time involved and frequency of agitation, the viscosity of the hydrated mucilage, the tenacity with which the mucilage adheres to the seeds as it is hydrated, the degree to which the mucilage is dispersed in the available water, and the degree to which the mucilage remains dispersed or separates, are all of considerable importance.

If the agitation of the seeds is reduced to a minimum in making the swelling factor determination, the result is an impaction of the seed-mucilage mass at the bottom of the cylinder with a considerable layer of supernatant water overlying the seeds. If the mass is agitated so as to give each seed and its mucilage content space and water in which to swell, the seeds are supported by larger areas of hydrated mucilage as well as by the viscosity of the dispersed mucilage. Again, if the mass is constantly and vigorously agitated in such a manner as to separate completely the mucilage from the seed and effect a homogeneous dispersion of the mucilage in the water, the viscosity of the mucilage and its rate of separation from the suspension will determine the depth to which the seeds sink in the cylinder. All the possible stages and their resultant swelling factor readings have been obtained by the author with the same lot of seed allowing for readings of swelling factors which are so widely divergent as to make them meaningless as regards the *Test for Quality* in the National Formulary. The widely divergent readings averaged in the collaborative reports on swelling factors presented by Burlage (4) suggest clearly the necessity of standardizing or completely revising the provisions of the swelling factor determination process.

The reproducible constants obtained by this author pertaining to percentage of mucilage content, swelling factors, and viscosities, show no direct relationship between the many possible swelling factors obtainable for a given lot of seed and the per cent of mucilage yielded by the seeds. Neither is the per cent of mucilage obtainable from a given lot of seed an indication of the viscosity of that mucilage. The wide variations exhibited by different lots of the same species of seed with regard to these important features make it possible so to misinterpret the values as to allow a poor grade of Plantago seed to be placed on the market as *P. psyllum*, e.g., *P. arenaria* No. 4, on the basis of a misleading swelling factor which meets the N. F. minimum requirements for that species, when by more exacting tests the seeds prove to have many inferior qualities.

It is not intended that the complete answer be given in this report as there are yet many factors involved concerning which the author has found no satisfactory answers. At the present writing the factor of the completeness of the hydration of the mucilage and the separation or layering of the mucilage dispersions, regardless of the viscosity, obtained from one sample of seed and no separation of the mucilage obtained from another seed has not been investigated. It should be pointed out that the accumulation of carbon dioxide resulting from the fermentation of the mucilage and the respiration of the seed which becomes entrapped in the mucilage when soaked in water over the test period is, unless controlled, sufficient to defeat the test entirely. It is of considerable interest in this connection that the seeds of *P. wrightiana* resisted fermentation for several weeks, at which time yeast colonies began to develop, and even resisted direct transplants of mycelial mats of *Aspergillus niger*. This would seem to indicate that there is present in *P. wrightiana* a substance which prevents or retards the growth of bacteria and fungi, whereas the other seeds fermented and became covered with molds within twenty-four to forty-eight hours after adding water.

SUMMARY

The present-day use of the mucilage separated from the seed of Plantago and other seeds requires a means of determining quantitatively the mucilage available from a given lot of seeds. A convenient means for this

determination is presented. From the data in Table I it is evident that the swelling factor is not indicative of the mucilage content, but is dependent on the viscosity of the mucilage formed and other related factors. It has been shown that the viscosity of the mucilage in water varies considerably in different lots of the same species of Plantago seed. It is suggested that more exacting test standards than now required be considered for the evaluation of psyllium seed. Two native Plantago seeds have been described and assayed. *Plantago wrightiana* yields a seed which in all respects compares favorably with the official seeds.

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Chemical Studies in Oklahoma Plants. VI. *Physalis mollis* Nuttall—A Plant Insecticide*,†

By LOYD E. HARRIS

Physalis mollis, commonly known as smooth ground cherry, has been used as a fly poison. A chemical investigation of this plant has been made and its effects upon flies have been studied. The plant is effective as a fly poison and the active constituent is probably an alkaloid which has been detected.

THE PLANT *Physalis mollis* belongs to the *Solanaceae* family. It is also known as smooth ground cherry and was described by Nuttall (1) in 1834. It grows widely throughout Oklahoma. During the early days of the state, and before the advent of prepared fly sprays, the fresh plant was used for the control of house flies. The leaves and stems were bruised, mixed with a little water and some sugar added. This was set around at convenient places and it was very effective in killing flies. A search of the literature revealed no information on the chemistry of this plant so this investigation was undertaken.

* Received Sept. 26, 1947, from The Ohio State University College of Pharmacy, Columbus.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.
† This is a preliminary report. Work is to be continued using the fresh plant when available.

EXPERIMENTAL

The leaves were collected from flowering plants in Greer County, Oklahoma, in June, 1946. They were carefully washed and spread out to dry in the shade. The air-dried material was reduced to a No. 20 powder in a Wiley mill.

Extraction with Selective Solvents.—Using two samples in Soxhlet Extractors and the solvents in the order listed, the following results were obtained:

Solvent	Per Cent Extractive
Petroleum ether (b. p. 30–60°)	0.898
Ether	0.639
Chloroform	1.78
Alcohol (dehydrated)	15.40
Solvent containing 50% alcohol and 50% water	4.41

Petroleum Ether Extract.—The petroleum ether extractive was redissolved in the solvent in order to separate a white substance that had appeared in the evaporation of the original extract. This was separated by filtration and dissolved in hot alcohol. Upon evaporation of the alcohol, a residue was obtained that was somewhat crystalline, but not uniformly so. Carbon tetrachloride was used in an attempt to obtain a better crystalline product but without success. A small amount of the material was heated to a temperature of 175° in a tube at 1-mm. pressure but none of it sublimed. A melting point determination gave an indefinite melting point of approximately 268°.

Alcohol Extract.—During the alcohol extraction, crystals were observed in the alcoholic extract. These crystals were separated and identified as potassium nitrate by the standard qualitative tests. All subsequent extractions with alcohol or water gave a yield of this salt that interfered with the attempts to purify other constituents. A nitrate determination was made on the aqueous extract from an air-dried sample using a method described by Klein (2). This showed the air-dried leaves to contain 2.4% of potassium nitrate.

The alcoholic extract was treated with hot water to obtain the water soluble fraction. The aqueous solution was tested with Benedict's qualitative solution. There was no reaction. After a small amount of hydrochloric acid was added, and the solution warmed, an insoluble substance precipitated After filtration, the filtrate gave a positive test with Benedict's reagent. This showed the presence of a glycoside. Its formula was not determined other than to establish the identity of the sugar as glucose by its optical activity and the formation of an osazone.

Chloroform Extract.— The chloroform extract was redissolved in chloroform and extracted with dilute aqueous sulfuric acid. The aqueous acid solution was made alkaline with ammonia and extracted with chloroform. This extraction process was repeated. The evaporation of the chloroform gave a very small amount of a white residue that was insoluble in water but soluble in chloroform and carbon tetrachloride.

In order to obtain more of this material, 100 Gm. of the powdered leaves were mixed with a menstruum of two volumes of chloroform and one volume of ether and then 10 cc. of ammonium hydroxide was added. The sample was allowed to macerate overnight and then extracted with chloroform using a Soxhlet extractor. The chloroform extract was then treated as before, except that the solution was used without evaporation. The residue obtained on the final evaporation represented 0.038% of the sample. The material contained a small amount of an oil-like substance that had a strong unpleasant odor. This was removed by washing with ether. The material, at this point, gave a melting point of 135–138°. The white residue, dissolved in 1% hydrochloric acid, gave a precipitate with the following alkaloidal reagents; Mayer's, phosphotungstic acid, and gold chloride. Negative tests were obtained with platinic chloride, ferric chloride, and picric acid.

Since the plant is in the same family as belladonna, it was thought possible that the substance might be atropine but Vitalli's test was negative. A purple color was obtained, however, by omitting the preliminary treatment with nitric acid. A similar color was obtained by using 90% sulfuric acid.

Biological Tests.—In order to determine which was the active portion of the plant, approximately 500 flies (*Musca domestica*) were divided into three test groups. Using diluted skim milk as the vehicle, the flies in one group were given the impure glycosidal material, another group was given about 4 mg. of the alkaloid and the third group was used as a control. After four hours the flies getting the glycoside were dead, and the other groups were not affected. These were allowed to continue overnight but they were still unaffected the next morning The control group was then given the skim milk mixed with the powdered leaves. This proved fatal to all of them. The group having the alkaloid remained unaffected. This showed the glycosidal material to be toxic while the alkaloid was nontoxic in the small dosage given.

SUMMARY

Physalis mollis Nuttall, is a plant that is indigenous to Oklahoma and has been used as a fly poison by residents of that state. Its toxic effect on the common house fly was demonstrated.

A glycoside was isolated in an impure form. This was found to be toxic to flies (*Musca domestica*). The glycone of the glycoside was identified as glucose. An alkaloid was isolated that showed some characteristic tests. It has a melting point of 135–138°. This substance was not toxic to flies in the small dosage used.

Potassium nitrate was found to be present in the amount of 2.4 per cent in the air dried leaves.

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Chemical Determination of Synthetic Estrogens. II. Application to Pharmaceutical Preparations of Diethylstilbestrol*

By SIDNEY GOTTLIEB

Methods are presented for the analysis of tablets, oil solutions, and cosmetic creams containing diethylstilbestrol by use of the nitrosophenol quinoid analysis previously reported.

In the previous paper (1) a method was proposed for the determination of a group of synthetic estrogens, consisting of nitrosation of the estrogen to the *o*-nitrosophenol and measurement of the intense color of the quinoid form of the *o*-nitrosophenol in alkaline solution. Data were presented for diethylstilbestrol, hexestrol, dienestrol, benzestrol, and meprane. It is the purpose of this paper to report on methods which have been developed to apply this reaction to various pharmaceutical preparations of diethylstilbestrol.

The U. S. P. XIII lists Diethylstilbestrol Tablets, Diethylstilbestrol Injection, and Diethylstilbestrol Capsules. The two latter forms are solutions of the estrogen in oil. Nonofficial forms of synthetic estrogens are in wide use. Creams, both vanishing and cold cream bases, containing synthetic estrogens, are examples of such preparations. Gynecological creams containing synthetic estrogens have also appeared on the market. Proposed methods for the analysis of such diethylstilbestrol preparations are described below under the headings Tablets, Oil Solutions, Cold Creams, and Vanishing Creams.

Materials

Buffer solution. To 800 cc. of glacial acetic acid add 150 cc. of 10% potassium hydroxide solution and 50 cc. of water, and mix well.

Saturated aqueous sodium nitrite solution.

Concentrated sulfuric acid (36 *N*).

Concentrated hydrochloric acid (12 *N*).

Alcoholic ammonium hydroxide solution. Mix 450 cc. anhydrous ethyl alcohol or isopropyl alcohol, 300 cc. of concentrated ammonium hydroxide (14 *N*), and 250 cc. of water.

Sodium hydroxide solution (1 *N*).

Sulfuric acid solution, 10%.

Alumina—activated alumina, 80–200 mesh.[1]

Silicic acid, C. P., precipitated.[1]

Isopropyl ether. Free 1 L. of the commercial chemical from peroxides by shaking with a solution made up from 150 Gm. of ferrous sulfate, 15 cc. of concentrated sulfuric acid, and 275 cc. of water. Following this treatment, wash the ether several times with distilled water and dry over anhydrous calcium chloride. It may be stored over calcium chloride and filtered before use. Do not use any ether that has been standing for more than a week.

Toluene, C. P.

Ethyl alcohol, 95%.

Tablets

Weigh accurately a portion of the powdered tablets equivalent to 15–20 mg. of diethylstilbestrol. Suspend the powder in a separatory funnel in 30 cc. of distilled water to which 5 drops of concentrated hydrochloric acid have been added. Extract this suspension twice with 25-cc. portions and three times with 10-cc. portions of peroxide-free isopropyl ether. Extract the combined isopropyl ether extracts first with 25 cc., then three times with 15-cc. portions of sodium hydroxide solution. Render the combined alkali extracts acid with dilute sulfuric acid and extract twice with 25-cc. portions and three times with 10-cc. portions of isopropyl ether. Filter the combined extracts through a dry filter and wash the filter with several small portions of isopropyl ether. Evaporate the ether to dryness and dissolve the residue in exactly 50 cc. of ethyl alcohol.

Take an aliquot of the final alcoholic solution equivalent to from 0.1 to 0.5 mg. of diethylstilbestrol and evaporate to dryness on the steam bath using a stream of air to remove the last traces of alcohol. Dissolve the residue in 5 cc. of buffer solution. Add 5 drops of concentrated sulfuric acid and 2 drops of saturated aqueous sodium nitrite solution. Allow this solution to stand thirty minutes with occasional mixing, then dilute to near 50 cc. with alcoholic ammonium hydroxide, cooling the volumetric flask in an ice bath as the alkali is added. Let stand thirty minutes, dilute to exactly 50 cc. with alcoholic ammonium hydroxide and filter a portion of this solution through a dry filter. De-

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[1] In this study, the alumina used was grade F-20 made by the Aluminum Ore Company, and the silicic acid was from Eimer and Amend, lot No. 403320. It should be emphasized that each lot of alumina and silicic acid used should first be standardized by passing a solution of the estrogen of known strength over a column of the adsorbent to determine whether or not complete recovery can be attained.

termine the amount of diethylstilbestrol in this filtrate by measuring its absorption at 440 mμ and comparing with a standard similarly treated, containing 0.2–0.3 mg. diethylstilbestrol, or by using a standard curve over the range 0.1 to 1.0 mg. diethylstilbestrol.

Oil Solutions

This method can be used for both oil-containing capsules and oil for injection in ampuls. The U. S. P. XIII specifications for removing the oil sample may be applied, using a sample containing about 5 mg. diethylstilbestrol, and dissolving the oil in 50 cc. of isopropyl ether. Proceed as described under "Tablets" above, beginning with "Extract the combined isopropyl ether extracts first with 25 cc., then three times with 15-cc. portions of sodium hydroxide solution."

Creams and Ointments

Under this heading are included vanishing and cold cream bases for skin application, and also various types of gynecological creams. It was necessary to develop separate methods for the cold cream base preparations (high oil/water ratio) and for the vanishing cream base (low oil/water ratio).

a. **Cold Cream Base.**—Accurately weigh an amount of cream containing 2–5 mg. of diethylstilbestrol. Transfer with water and toluene to a 500-cc. flask, using a total of 100 cc. toluene and 50 cc. of water. Add 1 cc. of concentrated hydrochloric acid, and reflux the mixture until a clear separation of the two layers is attained. This usually takes from twenty to forty minutes of moderate refluxing. Carefully separate the layers and shake the aqueous layer with three 25-cc. portions of toluene, and combine the toluene extracts with the original toluene layer. Prepare a 15- x 160-mm. adsorption column of activated alumina and wet with toluene. Pass the toluene solution through the column, followed in succession by 100 cc. of toluene and 100 cc. of isopropyl ether. Then run 100 cc. of 95% ethyl alcohol through the column, collect the alcohol and evaporate it to dryness. Dissolve the residue in 50 cc. of isopropyl ether and continue the analysis as described under "Tablets," beginning with "Extract the combined isopropyl ether extracts first with 25 cc., then three times with 15-cc. portions of sodium hydroxide solution."

b. **Vanishing Cream Base.**—Use the size of sample and the separation of the emulsion into an aqueous and a toluene phase as described under "Cold Cream Base." Use the same amount of concentrated hydrochloric acid. Pass the combined toluene extracts over a column prepared by intimately grinding 10 cc. methyl alcohol into 20 Gm. of anhydrous precipitated silicic acid. Make this mixture into a slurry with toluene and pour into a column 32 mm. wide. After the slurry runs nearly dry, put on the combined toluene extracts, followed in succession by 100 cc. of toluene and 200 cc. of isopropyl ether. Continue the analysis, using the 200-cc. isopropyl ether filtrate, as described under "Tablets," beginning with "Extract the combined isopropyl ether extracts first with 25 cc., then three times with 15-cc. portions of sodium hydroxide solution."

RESULTS AND DISCUSSION

Table I indicates the results of the analyses of several different preparations, both commercial samples and laboratory preparations.

The laboratory mixture of tablet powder base was made up of 90% corn starch, 3% $CaCO_3$, 3% talc, 2% lactose, and 2% sucrose. Diethylstilbestrol was thoroughly mixed with the powder so that it was 0.095% of the total. In general, no difficulties were encountered with tablet preparations.

The laboratory mixture of oil solution consisted of 0.1% diethylstilbestrol in a mixture of equal parts of U. S. P. Corn Oil, Cottonseed Oil, and Sesame Oil In preliminary work, an attempt had been made to include a chromatographic step, using an alumina column, since it was felt that the steroids from the oils would appear in the final residues for analysis. It was found that the diethylstilbestrol could be adsorbed from dry isopropyl ether onto alumina by washing the column with isopropyl ether and isopropyl ether-ethyl alcohol mixtures and the estrogen could finally be washed off in fairly pure state with 95% ethyl alcohol. However, since sterols in moderate amounts do not interfere with the nitrosophenol analysis, and since it was difficult to standardize different batches of alumina, this step was abandoned. It has been used occasionally in special cases where an interfering substance makes it necessary.

The cold cream laboratory mixture was made by melting together 350 Gm. lanolin, 350 Gm. mineral oil, 84 Gm. spermaceti, and 98 Gm. of beeswax at 60°. Seven-tenths gram naphthylamine, 1 Gm. β-ionone, 0.7 Gm. ethyl-*p*-hydroxybenzoate, and 100 mg. diethylstilbestrol were stirred into this melt and the whole was heated to 70°. Seven grams of borax, dissolved in 511 Gm. of water at 72°, was added to the other solution with constant stirring allowing the temperature to fall during the mixing to 45–50°, at which temperature it was bottled. It will be noted that the esters of *p*-hydroxy benzoic acid, which are common preservatives for cosmetic creams, do not interfere with the analysis, although a free phenolic group is present. When conventional shakeout procedures were used, it was found that these preservatives would interfere with the analysis, and also that very troublesome emulsions would be formed in the initial shakeouts. The refluxing with toluene affords a simple method of cleanly breaking the emulsion, and the subsequent adsorption step allowed essentially everything but the estrogen to be separated from the column. Particular attention was given to the cold creams, because the great majority of estrogen creams appearing on the market are of this type. This type of separation could readily be applied in other cases where a

TABLE I.—DIETHYLSTILBESTROL ANALYSES ON VARIOUS PHARMACEUTICAL PREPARATIONS

Dosage Form	Sample	Amount of Stilbestrol Known or Declared, Mg.	% of Stilbestrol Known or Declared	Amount of Stilbestrol Found, Mg.	% of Known or Declared Amount Found
Tablets	1. Laboratory mixture	4.80	0.095 (*W/W*)	4.73	98.6
	2. Replicate of 1	4.80	0.095 (*W/W*)	4.67	97.2
	3. Replicate of 1	4.80	0.095 (*W/W*)	4.70	98.0
	4. Comm. sample—1 mg./tab.	15.00	2.5 (*W/W*)	14.86	99.0
	5. Comm. sample—0.5 mg./tab.	20.00	0.29 (*W/W*)	19.61	98.1
Oil Solutions	1. Laboratory mixture	4.68	0.1 (*W/V*)	4.62	98.8
	2. Replicate of 1	4.68	0.1 (*W/V*)	4.66	99.6
	3. Replicate of 1	4.68	0.1 (*W/V*)	4.64	99.2
	4. Capsules, 5 mg./cap.	25.00	... (*W/V*)	24.32	97.4
	5. Oil for injection, 5 mg./cc.	5.00	0.5 (*W/V*)	4.93	98.6
Cold Creams	1. Laboratory mixture	2.00	0.071 (*W/W*)	1.94	97.0
	2. Replicate of 1	2.00	0.071 (*W/W*)	1.96	98.1
	3. Replicate of 1	2.00	0.071 (*W/W*)	1.93	96.6
	4. Comm. sample—2.5 mg./oz.	2.50	0.089 (*W/W*)	2.42	96.8
	5. Comm. sample—2.5 mg./oz.	2.50	0.089 (*W/W*)	2.45	98.0
Vanishing Creams	1. Laboratory mixture	4.00	0.143 (*W/W*)	3.91	97.8
	2. Replicate of 1	4.00	0.143 (*W/W*)	3.89	97.3
	3. Replicate of 1	4.00	0.143 (*W/W*)	3.93	98.2
	4. Comm. sample—2 mg./oz.	2.00	0.071 (*W/W*)	1.96	98.0
	5. Comm. sample—2.5 mg./oz.	2.50	0.089 (*W/W*)	2.39	95.6

minute amount of a substance to be determined is incorporated in a large amount of cream.

The laboratory preparation of vanishing cream was carried out by adding a solution of 98 Gm. of glycerin, 9.24 Gm. of potassium hydroxide, and 5.6 Gm. of triethanolamine in 700 cc. of water heated to 85° to a solution of 200 mg. stilbestrol in 185 Gm of stearic acid heated to 85°. This addition was carried out under constant and vigorous stirring. When this mixture had cooled to 50°, a dispersion of 28 Gm. of glyceryl monostearate, 2 Gm. of β-ionone, 0.7 Gm. of butyl-*p*-hydroxy benzoate in 374 Gm. of water was thoroughly incorporated and the mixture cooled and bottled. When the method designed for the cold cream base was applied to this preparation, very low recoveries were obtained, apparently due to the solubility of the estrogen in the large amount of stearic acid. In every method tried for separating the stearic acid from the estrogen, the stearic acid carried along appreciable amounts of stilbestrol. Several adsorbents, including talc, sucrose, starch, and celite, were ineffective. The method finally adopted utilized the partition chromatographic technique originally discussed by Martin and Synge (2). The method is not entirely suitable for routine analysis, since the preparation of the column entails considerable time and care, and since different batches of anhydrous precipitated silicic acid will vary greatly and hence must be carefully standardized for the particular separation contemplated. The method is reported here, however, because it does give good results when carefully used.

Preliminary experiments indicate that dienestrol, benzestrol, hexestrol, monomestrol, and meprane can be readily determined in tablet and oil preparations by the method outlined above. The meprane must first be hydrolyzed to the free phenol. Their determination in the cosmetic and gynecological creams is yet to be made. It is to be expected that the structural variations in these compounds would bring about differences in their behavior on the adsorbents utilized in the analysis of creams. However, a determination of a gynecological cream containing dienestrol has been successfully carried out by the method outlined for cold creams.

SUMMARY

Methods are presented for the analysis of diethylstilbestrol in tablets, oil solutions, and creams, utilizing the formation of nitrosophenol quinoid compounds. Results indicate the method can be successfully applied to these preparations.

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A Photoelectric Colorimetric Assay for Gentian Violet by Comparison with a Standard Powder*,†

By JOSEPH D. MC INTYRE‡ and ARTHUR E. JAMES

A colorimetric photoelectric quantitative method has been developed whereby the gentian violet content of tablets may be determined by comparison with a standard reference powder. Samples of gentian violet may be compared with each other.

THE official pharmaceutical compendiums in the United States and in several foreign countries are reflecting the growing importance and acceptance of gentian violet. Since 1925 New and Nonofficial Remedies (1) has included it as an acceptable therapeutic agent. The Swiss Pharmacopœia of 1933 (2) listed gentian violet under the title of "Methylrosaniline Chloratum." It was introduced into the National Formulary VI (3), in 1936, under the title of "Methyl-rosaniline," using as synonyms the terms Gentian Violet, Methyl Violet, and Crystal Violet. The National Formulary VII (4) carried Methyl-rosaniline Chloride solution while the current edition, National Formulary VIII (5), continues to list the solution and has added a jelly. The United States Pharmacopœia XII, issued in 1940, (6) adopted Methyl Rosaniline Chloride. The Extra Pharmacopœia of Great Britain (7) adopted Medicinal Gentian Violet in 1936 under the title of Methyl Violet. Notwithstanding the increased use of gentian violet none of the official reference books contains directions for its assay.

Albert (8) has recently suggested a gravimetric method for the assay of gentian violet in which a suitable weight of sample dissolved in water is heated with 1 *N* NaOH. The resulting insoluble carbinol is extracted with benzene. After filtering, the benzene extract is evaporated and the residue is dried to constant weight. Each gram of carbinol is equivalent to a fixed weight of gentian violet. However, the Albert procedure has proved unsatisfactory in the hands of competent analysts when applied to the assay of tablets of gentian violet. The carbinol is so tenaciously adsorbed on the tablet excipients that it is very difficult to extract. Conn (9) and others (10) have suggested a volumetric assay for gentian violet using a standard solution of titanous chloride, $TiCl_3$. Titration with this chemical has several limitations including the necessity of excluding air from the solution.

In view of the intense color of aqueous solutions of gentian violet and of the general acceptance of photoelectric colorimetric methods for rapid analysis this type of assay has been studied in our laboratories and has been found to give satisfactory results.

EXPERIMENTAL[1]

Preparation of a Standing Graph for Comparison.—A sample of U. S. P. gentian violet[2] was dried to constant weight in a vacuum desiccator over concentrated sulfuric acid. Exactly 0.5 Gm. of this powder was dissolved in distilled water and diluted to a liter. Separate dilutions of this solution containing from 0.50 to 5.0 mg. of gentian violet per liter were prepared. Experiment indicated that the maximum absorption of U. S. P. gentian violet solutions is at 586 mμ. A light filter with maximum transmission between 500 and 570 mμ was used. With a Klett–Summerson photoelectric colorimeter[3] the transmission of the various solutions was determined and read on the logarithmic scale of the instrument. A typical set of data is as follows:

Concentration of Gentian Violet, Mg./L.	Colorimeter Reading
5.08	320
4.50	286
4.00	254
3.50	224
3.00	194
2.50	160
2.00	128
1.66	107
1.25	79
1.00	65
0.50	32

* Received Oct. 7, 1947, from the Research Laboratories of Dr. D. Jayne & Son Branch, Sterling Drug, Inc., and Temple University School of Pharmacy, Philadelphia.

† The powder accepted as standard in this investigation was supplied by the National Aniline Division of the Allied Chemical & Dye Corporation, Buffalo, N. Y. It is suggested that the U. S. P. Revision Committee establish a standard reference powder.

‡ Deceased Jan. 25, 1948.

[1] Acknowledgment is made to Joseph Marlino of Dr. D. Jayne & Son Branch, Sterling Drug, Inc., for checking experimental data shown herewith.

[2] See footnote indicated by dagger (†)

[3] Any suitable photoelectric colorimeter may be used.

The above data are transcribed to graphical form in Fig. 1. The concentration of gentian violet is plotted as abscissa and the corresponding colorimetric readings as ordinate.

Assay of Individual Tablets Containing Gentian Violet.—Individual enteric coated tablets claimed to contain approximately 25 mg. of gentian violet were assayed as follows. The outer sugar coating and enteric coating were removed by solution in distilled water. The nucleus of the tablet was pulverized after drying and the resulting powder dissolved in distilled water and made up to a volume of 1 L. Suitable dilutions were made to bring the concentration within the range of the colorimeter scale. The transmission of each solution was measured in the colorimeter and the corresponding concentrations of gentian violet interpolated from the standard graph. Typical results were as follows:

Colorimeter Reading	Gentian Violet, Mg.
158	24.70
157	24.65
147	23.00
156	24.67
153	24.00
143	22.30
158	24.70
151	23.50
150	23.50
144	22.30
	Av. 23.73 mg./tablet

Assay of Lots of 20 Tablets of Gentian Violet.—After suitable preparation and dilution the gentian violet content of each lot was determined colorimetrically. Typical data obtained were as follows:

Manufacturer or Source of Tablets	Labelled Content of Gentian Violet/Tablet, Mg.	Amount of Gentian Violet Found/Tablet, Mg.
A for adults	25	23.5, 23.4, 22.6
B for adults	25	23.5, 23.5, 22.6
A for children	9.7	8.5, 8.5, 8.0
C for children	9.7	8.5, 8.6, 8.3
A uncoated tablet nuclei		27.5, 28.0, 28.5

DISCUSSION OF RESULTS

It was found expedient to dry gentian violet to constant weight in a vacuum desiccator over concentrated sulfuric acid. Efforts to dry it by heating in an oven at 110°, as specified in the U. S. P., required several weeks before attaining constant weight. The reason for this appears to be due to the very gradual loss of water of hydration. Several hydrates—a tri, and hexa or nona hydrate—of crystal violet have been reported (8).

The calibration graph as shown in Fig. 1 shows close adherence to Beer's Law indicating that the color intensity is in direct proportion to the concentration of gentian violet.

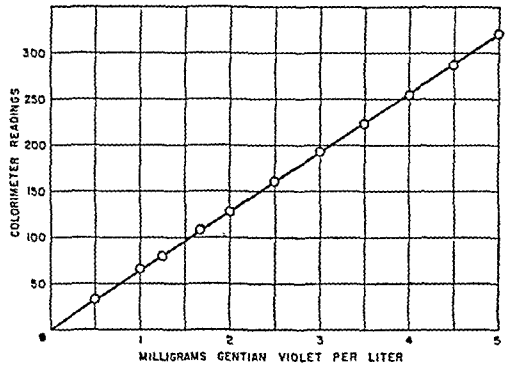

Fig. 1.—House Reference Standard. Dried to constant weight.

Aqueous solutions of gentian violet were found to be stable when exposed to daylight for several months. Solutions of tablets made by some manufacturers were not stable toward light if exposed for several hours. However, the solutions of tablets obtained from other manufacturers were stable. This difference in behavior is attributed to differences in tablet excipients.

It is noteworthy that, with the exception of the uncoated tablet nuclei, the results of the above assays show less gentian violet than the labelled content. It is believed that this is due to the presence of small amounts of reducing sugars which are present in both the enteric coating and in the excipient of the tablets.

For laboratory control of manufacturing processes, it is desirable that a rapid assay method to determine the content of gentian violet be developed. The colorimetric assay proposed herewith fulfills this need. It is applicable to the assay of samples of gentian violet and to the assay of tablets containing this product. Either single tablets or lots of 20 may be used.

As the U. S. P. does not at present have an assay method to determine the potency of gentian violet the authors of this paper believe that it would be in the interest of public health for the Revision Committee to establish a standard reference powder.

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A Histological Study of the Glandular Hairs of Lophanthus Anisatus Benth.*

By ROBERT L. VAN HORNE† and LOUIS C. ZOPF‡

Results of a study of the morphological development of the glandular hairs of *Lophanthus anisatus* are presented. Microphotographs illustrate the steps in the cell division which lead from a single epidermal cell to the mature eight or more celled volatile oil-producing glandular hair. Tissue tests demonstrate that cutin and pectic materials are present in the outer wall of the glandular hairs.

THE cultivation of mint plants as farm crops has been practiced in the United States, as well as abroad, for many years. The United States Department of Agriculture has carried on intensive investigations regarding growing conditions, methods of harvesting the plants, and the problems attendant to extracting the volatile oils. Van Fleet (1), and Sievers (2), have studied thoroughly the effects of various cultivating conditions on the peppermint plant which is recommended as a profitable farm crop.

More recently, several workers have investigated mint plants from the standpoint of the chemical composition and possible therapeutic use of their volatile oils. In the course of this work, attention was directed to the location and structure of the cells producing the volatile oils. Hocking and Edwards (3) attempted to correlate the numbers and dimensions of the glandular scales of *Mentha piperita* with the amount of oil produced in the species. Some interesting comparisons of the volume of oil produced by species of the plants grown in Florida and in Indiana resulted, but little correlation was found to exist between the number of oil cells and the volume of oil produced.

Ripert (4), in a discussion of the location of the oil-producing tissues of peppermint, stated that the volatile oil develops in the protoplasm of the epidermal foot cell, and that this cell is the parent cell of the glandular scales. He concluded that the oil is a waste product of metabolism.

Murthy (5), of the Department of Botany, Central College, Bangalore, India, studied the development of the glandular hairs of *Orthosiphon stamineus*, another member of the *Labiatae*. He illustrated, by drawings, how the hair first appears as an enlarged epidermal cell which divides periclinally to form a stalk cell and a head cell. The stalk cell then divides periclinally again to form a middle cell and a basal cell. The head cell divides anticlinally into two cells. These cells divide further into four cells and again into eight. The volatile oil is secreted beneath the cuticle which is distended to form a bladder which ruptures when the pressure of the oil becomes too great for the cuticle to withstand, thus liberating the oil into the atmosphere.

Recently, Politis (6) published his observations regarding the secretion of essential oils in plants belonging to the *Labiatae* and other volatile oil-producing plants. He asserts that the cuticle on the glandular hairs raises to form the balloon-shaped pocket into which the essential oil is secreted.

This investigation of *Lophanthus anisatus* was undertaken to show by the use of photomicrographs and drawings the sequence of development of the glandular hairs.

The selection of the subject plant was influenced by the fact that it was already growing in the Pharmacy-Botany gardens of the State University of Iowa. It had been planted there from seed furnished in 1942 by Mr. Frank Pellett, of the Pellett horticultural gardens of Atlantic, Iowa. Pellett had published several papers on the usefulness of the plant as an important source of nectar for honey bees (7–9).

Lophanthus anisatus Bentham, commonly

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† Fellow, American Foundation for Pharmaceutical Education.
‡ Professor of Pharmacy, College of Pharmacy, State University of Iowa.

called anise hyssop because of its fragrant anise-like odor, is a slender, tall, herbaceous plant which may reach a height of one meter or more, Fig. 1. It is known by several other botanical synonyms, *Agastache anethiodora* Nutt. and *Agastache foeniculum* Pursh being the more common of them. In addition to the common name of anise hyssop, it is also called *Hyssopus ambiguous* but is not to be confused with *Hyssopus officinalis* which is the well-known cultivated variety of the hyssop. Its habitat ranges from Illinois westward to the foothills of the Rocky Mountains in Colorado and northward to Manitoba.

The plant in bloom is characterized by its fragrant spike of blue flowers which grow in a dense head and attract all sorts of insects from the moment the flowers open until blooming ceases in late summer.

Fig. 1.—*Lophanthus anisatus*, Benth.

It produces a volatile oil which has been analyzed by Polak and Hixon (10) recently and which was also reported on in 1898 and 1913 by Schimmel and Company (11, 12). We did not attempt an analysis of the oil in our work.

Fig. 2.—Longitudinal section through flower bud. All three types of hairs are visible on outer surface of calyx.

EXPERIMENTAL

Since we wished to trace the stages in the growth and development of the glandular hairs, we collected specimens at intervals allowing for various stages of maturity. The first collections were made on April 7, 1946, and collections of the upper leaves were made at intervals of about two weeks until the first week of June when the flowers opened. As soon as the material was collected, it was killed and fixed in a formalin–acetic acid–alcohol mixture. This was found to be a good killing fluid for leaves but the flowers, because of their succulent nature, were more satisfactorily fixed in a modified Nawaschin solution. Paraffin was used to embed the material for sectioning on the rotary microtome.

Safranin and fast green were employed to stain the sections preparatory to examining them for the glandular hairs. These stains gave good contrast in the photomicrographs. During the process of staining the tissues we became interested in determining, if possible, the nature of the material which forms the balloon-like sacs about the mature glandular hairs. As mentioned before, Politis stated that the cuticle about the hairs becomes distended into the balloon-like shape when the cells secrete their volatile oil.

Using prepared sections from killed and fixed material, we tried a number of tests to determine whether the material composing the outer wall is really cutin or a mixture of substances such as pectic

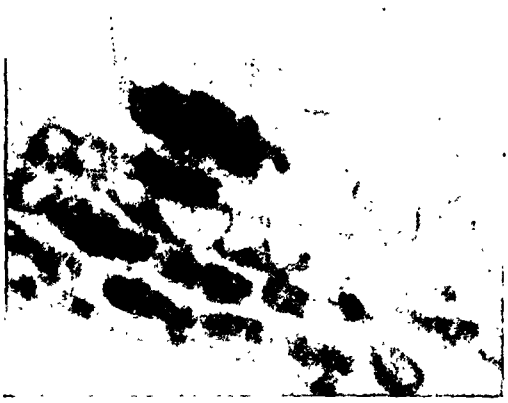

Fig. 3.—Mature glandular hair with collapsed cuticle. Nonglandular hair at left.

materials, cellulose, and cutin. By immersing sections of the flower heads, which had numerous glandular hairs on the corolla and calyx, in 50% chromic acid solution in the cold, all the material but the envelope surrounding the glandular hair, and the cuticle of the epidermal layer, was dissolved away. Upon staining the residue left on the slide with Sudan IV, a red color was produced in the cuticle of the epidermis and also in the sacs which had enveloped the hairs, indicating the probability that at least some of the material in the sac is cutin. It is not reasonable to expect that cutin alone could expand or stretch sufficiently to maintain its intact character as the glandular hairs grow from the one-celled stage to maturity. By the use of Ruthenium Red in a very dilute aqueous solution, we were successful in demonstrating the presence of pectic materials in freshly cut sections bearing glandular hairs. It is apparent then that the sac surrounding each glandular hair is composed of both cutin and pectic material.

DISCUSSION

Three types of hairs occur on the leaves and flower parts of *Lophanthus anisatus*. Most numerous is the nonglandular, papillose, several-celled type of hair. These are abundant on the lower epidermis of the leaf and also on the outer surfaces of the corolla and calyx, Fig. 2. The number of cells varies from one to three, or rarely, four. The apical cell is almost always sharply pointed. In many of these hairs, the nucleus is visible in the young plant, and stains red with rapid safranin. These hairs are usually straight but may recurve somewhat, particularly those found on the lower leaf surfaces.

A second type of hair is composed of a basal cell, a stalk cell, and one or two glandular head cells. A prominent cuticle surrounds the head cells and they stain deeply with safranin in the flowering plant, but are unstained in the plants which have not yet reached the stage of anthesis. They occur in lesser numbers than the nonglandular hairs but in the same areas. In some cases they may be found in scattered points on the upper epidermis of the leaf but their most common location is on the lower leaf surfaces and on the outer surfaces of the calyx and corolla. These one- or two-celled glandular hairs are present in all stages of formation of the third type of hair, the multicelled, balloon-shaped glandular hairs, and appear to be distinct from the latter Fig. 3.

This type is supported by a unicellular stalk and arises from a large epidermal cell surrounded by a number of much smaller epidermal cells. The ontogeny of the glandular hairs is the main subject of this paper.

As observed in the very young leaf and flower, the development of the typical multicellular glandular hair begins with the enlargement of an epidermal cell. Such an epidermal cell in the young tissue has not yet developed a distinct cuticle and its outer

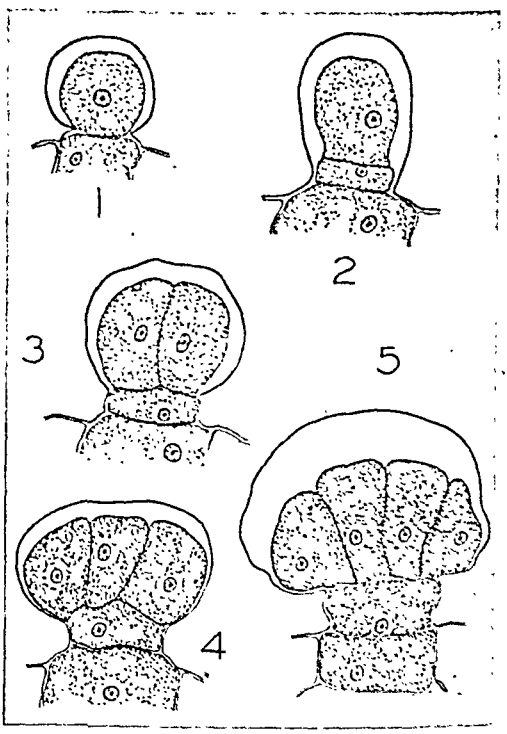

Fig. 4.—Drawings of various stages of development of glandular hairs. *1*—First stage after initial division of epidermal cell. *2*—Head cell differentiated from stalk cell. *3, 4*—Successive stages in cell division in head results in mature glandular hair with eight or more secreting cells—*5*.

wall is still elastic. The increase in cell contents stretches the upper wall so that the enlargement of the cell is in a direction external to the epidermal layer. Subsequent division of the enlarged epidermal cell gives rise to a daughter cell, Fig. 4. Upon completion of the division of the epidermal cell and the subsequent enlargement of the daughter cell, a periclinal division of the new cell occurs and the result is the head cell and the stalk cell, which latter retains its identity throughout the remainder of the glandular hair formation. By anticlinal division, the head cell gives rise to two head cells, Fig. 5, and continued anticlinal division results in the formation of first, four head cells and then eight, with rarely, ten or twelve cells, Fig. 6, occurring in the mature glandular hair.

In this process of division, the cuticle surrounding the entire glandular hair enlarges so that the mature hair has a balloon-shaped cuticle intact around the whole of the head.

As the secretion of the volatile oil commences in the glandular hair, the cuticle becomes distended and may eventually burst, Fig. 3, releasing the volatile contents into the atmosphere (4, 13). Hanstein (14) states that the cuticle may be regenerated in some cases and the process of secretion may be repeated in the same glandular hair. Ripert (4) concurs in this conclusion.

Fig. 6.—Cross section through head of twelve-celled glandular hair. Stalk cell is out of plane of focus.

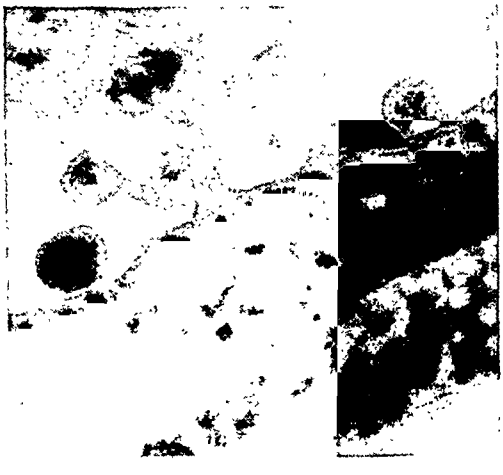

Fig. 5.—Two-celled glandular hair (right) and 4-celled hair (left) of *L. anisatus*. The stalk cell of each hair is also visible.

There are various opinions as to the reason for the formation of the oil and also as to the location of the initial secretion. Haberlandt (13) believed that the secretion was derived from raw materials present in the original cell contents, and in his later work Politis concluded that glycosides were the precursors of the essential oils. The fact that in staining glandular hairs in anise hyssop in the fresh condition with Sudan IV, numerous globules and granular inclusions in the head cells were observed, leads one to conclude that the oil may be formed in the cells of the glandular hair. Also, in dehydrated material, there remained granular material which stained deeply with safranin and made observation of the nuclei difficult. Such stained material made it impossible to obtain clear resolution of the individual cells for photomicrographs.

Photographs of the various phases of the cell division in the development of the glandular hairs were made to show clearly the steps in the process. It proved difficult to achieve clear-cut pictures due to the high magnification required and the accompanying lack of depth of focus. However, it is hoped that the pictures will aid in the clear understanding of the steps in the cell division.

SUMMARY

1. The development of the secretory cells which produce the volatile oil in *Lophanthus anisatus* has been observed and recorded by the use of photomicrographs.

2. The sequence of the development of the glandular hairs starts with the enlargement of an epidermal cell; this divides into a basal cell and a stalk cell. The stalk cell divides periclinally to give a head cell and a basal stalk cell. The head cell divides anticlinally into first two, then four, and finally eight cells. Some further development may

occur in a few hairs giving rise to as many as ten or twelve cells in the head. This development is therefore similar to that observed for a number of other species.

3. The composition of the cuticle surrounding the glandular hair was determined by tissue tests to be cutin, pectic materials, and possibly some other undetermined material.

4. The volatile oil is apparently secreted in the glandular hair causing the cuticle surrounding the hair to become distended with the accumulating secretions and to rupture.

5. Drawings to supplement the photographs depict the steps in the cell division in the glandular hairs.

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The Assay of Bacitracin*

By GRANT D. DARKER, HELEN B. BROWN, ALFRED H. FREE,† BARBARA BIRO, and JOHN T. GOORLEY

Two procedures, turbidimetric and cylinder plate, are reported for the assay of bacitracin. Two standard curves based on the Food and Drug Administration and the Cooper–Woodman methods are shown for the cylinder plate assay. The mathematical relationship between the antibiotic concentration and the zone of inhibition of bacitracin is discussed.

IN 1945 Johnson, Anker, and Meleney (1) described an antibiotic which they named bacitracin. It is produced by an organism of the *Bacillus subtilis* group which was isolated from a contaminated wound. A serial dilution method of assay was described in which a stock strain of a Group A hemolytic streptococcus was used as the test organism. A tentative standard unit was defined by this technique as "the amount which when diluted 1:1024 in a series of twofold dilutions in 2 cc. beef infusion broth, completely inhibits the growth of a stock strain of Group A hemolytic streptococcus when the inoculum used to seed the tubes is 0.1 cc. of a 10^{-2} dilution of an overnight culture in blood broth."

The present paper describes other assay techniques developed in our laboratory during the past two years. We have adopted the original definition of a unit of bacitracin and have checked our working standards by the serial dilution technique. Since it was not convenient to use the serial dilution method for a large number of assays in routine testing, two techniques already in use for penicillin assay, the turbidimetric and cylinder plate procedures, were modified for the assay of bacitracin. The cylinder plate method has been used routinely in our laboratories.

THE TURBIDIMETRIC ASSAY

The turbidimetric technique is a direct application of the procedure we have used for penicillin (unpublished data) and similar to that described by McMahan (2). It is rapid and sensitive from 0.2 to 0.8 unit per ml. of the sample. It is accurate to within ±20%. *Staphylococcus aureus* (F. D. A. 209-P) is grown in Bacto-yeast broth. The retardation of growth of the test organism obtained with a series of dilutions of the assay sample is compared with that obtained with a standard bacitracin solution. The tubes are incubated in a water bath at 38° for four and one-half hours when growth is stopped by steaming. The turbidity is estimated.

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by determining the per cent transmittance in a Coleman Universal Spectrophotometer at a wave length of 600 mμ. The amount of antibiotic activity, as measured by the turbidity, is given by the difference (Ts − Tb), between the growth obtained with the bacitracin sample (Ts) and that obtained with the organism alone (Tb). This in turn is compared to the inhibition obtained with a standard solution of bacitracin. Strictly sterile technique need not be observed. A typical curve is given in Fig. 1.

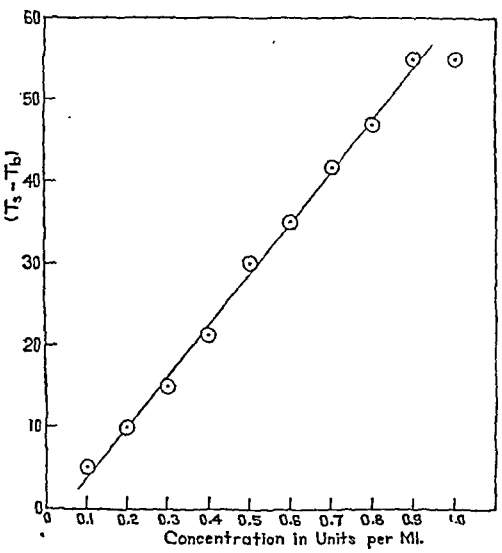

Fig. 1.—Turbidimetric assay. Ts−Tb *versus* bacitracin concentration.

Technique for Turbidimetric Method.—The assay as carried out in our laboratory is as follows: A suitable amount of sterile Bacto-yeast broth is inoculated with an 18-hour culture of *S. aureus*, 1 ml. per 100 ml. of broth. This is mixed thoroughly and dispensed into sterile test tubes in such amounts that the broth plus sample will be 10 ml. The standard solution is diluted with sterile uninoculated broth to a concentration of 1 unit per ml. The samples are diluted in the same way. These assay samples should have a *p*H between 4 and 6, contain no rapidly growing organisms, be clear, and have only slight color. There should be no organic solvents present as these will inhibit the growth of the bacteria. The inoculated broth is put into the tubes first. The diluted standard or samples are added by pipette, the tubes stoppered and immediately mixed. The series is set up so that from 0.1- to 1.0-ml. amounts are used in intervals of 0.1 ml. Blanks are made of uninoculated broth and control tubes of the inoculated broth alone. The racks of tubes are placed in a water bath at 38° for four and one-half hours. The racks of tubes are removed and steamed for ten minutes in the autoclave. The tubes are cooled, shaken, and read in the Coleman Spectrophotometer at a wave length of 600 mμ. The machine is set at 100% transmittance with the uninoculated broth blank. The difference between the per cent transmittance of the reading of any one tube minus the reading of the tube with *S. aureus* alone, (Ts − Tb), is a measure of the antibiotic concentration in that tube. The standard curve is run each day and the strength of the assay samples is taken from that curve.

THE CYLINDER PLATE ASSAY

The cylinder plate method is a modification of that employed for penicillin (3, 4). It is an adaption of the assay method of Schmidt and Moyer (4) in which the same organism, broth, agar, and buffer are used. It is suitable for solutions containing 1.5 to 8 units of bacitracin per ml. within an accuracy of ±15%. When the directions for the penicillin assay are followed precisely with the substitution of bacitracin for penicillin, small zones of inhibition are obtained and the variation of the zone diameters with variable concentrations of bacitracin is quite small. If the inoculated plates, with the cylinders holding the test solutions, are kept at 4° for six to ten hours prior to incubation, there is an increase in the zone size and greater variation in the zone diameter with varying concentrations of bacitracin. Refrigeration is necessary because the diffusion rate for bacitracin is lower than that for penicillin (see below).

Technique for Cylinder Plate Method.—The cylinder plate assay method is carried out in our laboratory as follows: An eighteen-hour culture of *S. aureus* (F. D. A. 209-P) is prepared in a yeast-beef broth. The frozen or dried bacitracin standard is diluted with 0.1 *M* phosphate buffer, *p*H 6.0, to give a working standard of 2.5 units per ml. All unknown samples are diluted with phosphate buffer to give a concentration of approximately 2.5 units per ml. When interfering substances such as alcohols are present in the samples, a dilution of at least 1:20 is necessary for accurate analyses. Plates are prepared by adding 22 ml. of nutrient agar to each, and are inoculated by flooding with 4 ml. of inoculated (0.8 ml. of eighteen-hour inoculum per 100 ml. melted agar) nutrient agar. The inoculated plates are kept at 4° until just before the cylinders are dropped in place. This prevents preliminary growth of the organism. Five cylinders are placed on each inoculated agar plate. Three of these are filled with the unknown sample and two with the standard. The plates are now refrigerated for nine to ten hours at 4° and then are placed in a 37° incubator for seven to eight hours. The plates are finally removed to the laboratory and the zones of inhibition are measured.

The Standard Curves.—The data for the standard curves were obtained from two samples of bacitracin which were standardized by the serial dilution method. In each test the working standard was diluted to give a range of solutions within the limits 0.5 and 40.0 units per ml. In all, seven series were

prepared, three with one standard and four with the other. On each plate two cups were filled with a 2.5 units per ml. solution and three with another concentration. Zones of inhibition were thus established for various concentrations of the standard in comparison with the 2.5 units per ml. solution. In tabulating the results, experimental values from plates showing the same zones of inhibition of the 2.5 units per ml. standard were segregated. Thus several series of data were established under the same experimental conditions against values of the inhibition zone of 2.5 units per ml. ranging from 15.0 to 18.0 mm. As the majority of the readings of the zones of inhibition of the 2.5 units per ml. concentration was in the 17.0 mm. series, this figure was selected for the basic standard curves. Table I shows two of the series with all of the experimental data for plates on which the 2.5 units per ml. standard gave zones of inhibition of 16.0 mm. (column 2) and 17.0 mm. (column 3), respectively. Column 1 gives the concentration in units per ml., and columns 4 and 5 record the numbers of readings on which columns 2 and 3, respectively, are based. The two columns to the right represent derived data used in the development of the Cooper–Woodman curve discussed later.

larger than that of the plate standard the difference is added to the zone size of the 2.5 units per ml. standard on the standard curve. This gives a corrected value for the unknown. For example, the unknown is 17.3 mm. and the plate standard is 16.5 mm., the difference, 0.8 mm., is added to 17.0 mm. to give a corrected value of 17.8 for the unknown. If the zone size for the unknown is smaller than that of the plate standard the difference is subtracted from the zone size of the 2.5 units per ml. standard on the standard curve. Thus, if the unknown is 15.3 mm. and the standard is 16.5 mm., the difference, 1.2 mm., is subtracted from 17.0 mm. to give a corrected value of 15.8 mm. for the unknown. Then for the corrected values of the zone size of the unknown the corresponding concentrations of bacitracin are read off the standard curve.

A series of standard curves has been plotted in our studies from experimental data assembled against readings of the 2.5 units per ml. standard ranging from 15.0 to 18.0 mm. All of these curves are essentially parallel. From a sufficiently large number of curves of this type it would be possible to read the value of the unknown without correction directly from the standard curve showing the same inhibition of the 2.5 units per ml. standard as the standard on

TABLE I.—RELATIONSHIP BETWEEN THE CONCENTRATION OF ANTIBIOTIC (m_0) AND THE ZONE OF INHIBITION ($2x + d$)[a] IN COMPARISON WITH 16.0- AND 17.0-MM. ZONES OF INHIBITION OF THE 2.5 U./ML. STANDARD

Units per Ml. (m_0)	Av. Zone of Inhibition ($2x + d$) against 2.5 U./Ml. Standard of 16.0 Mm.	17.0 Mm.	No. of Readings against 2.5 U./Ml. Standard of 16.0 Mm.	17.0 Mm.	Based on a 2.5 U./Ml. Standard Zone of 17.0 Mm. x	x^2
0.5	8.0	10.1	6	12	2.3	5.3
1.0	11.8	13.3	9	24	3.9	15.2
1.5	13.5	14.6	3	60	4.55	20.7
2.0	15.2	15.7	6	42	5.1	26.0
2.5	*16.0*	*17.0*	62	254	5.75	33.1
2.75	....	17.3	..	21	5.9	34.8
3.0	16.5	17.4	15	24	5.95	35.4
3.5	..	18.3	..	18	6.4	41.0
4.0	17.5	18.8	9	51	6.65	44.2
5.0	19.0	..	12	..	..	..
6.0	19.2	20.3	9	21	7.4	54.8
8.0	20.6	21.3	6	33	7.9	62.4
10.0	21.8	22.5	6	33	8.5	72.3
20.0	23.4	24.6	6	36	9.55	91.2
31.4	..	25.2	..	3	9.85	97.0
32.5	25.3	26.0	6	3	10.25	105.1

[a] d represents the inside diameter of the assay cylinder, in this example equal to 5.5 mm., and x represents the distance from the inner face of the cylinder to the edge of the zone of inhibition.

Figure 2 represents a standard curve of bacitracin determined according to the technique outlined by the Food and Drug Administration (5, p. 12133) for the assay of penicillin. It is based on the figures given in columns 1 and 3 of Table I for a standard zone of 17.0 mm. against a 2.5 units per ml. standard. In this graph on 3-cycle semilogarithmic paper, the diameter of the zone of inhibition has been plotted as the abscissa on the linear scale and the concentration of bacitracin as the ordinate on the logarithmic scale. To estimate the units in an unknown sample from a curve of this type the average zone sizes of the sample and the standard are calculated for the test plate. If the average zone size for the unknown sample is

the test plate. Since these standard curves on semilogarithmic paper are straight lines they represent the same type of variation expressed by the formula $y = a \cdot 10^{bF(x)}$. Log concentration or log $m_0 = \log a + bF(x)$, where $\log a$ represents the intercept on the y axis and b is the slope for any particular value of $F(x)$, in this instance $(2x + d)$. Therefore $\log m_0 - \log a \propto (2x + d)$. From this formula b may be determined for a known point and then corresponding values of m_0 may be calculated for various values of $(2x + d)$. Thus a chart may be prepared for direct reading of m_0 against any value of the zone of inhibition of the unknown solution. It is actually possible to construct a chart from two care-

fully determined points on a standard curve although in practice it is customary to establish a wide range of points in order to determine the useful limits of the curve.

Cooper and Woodman (6) have utilized the general formula in another form to express the relationship between the concentration of the antibiotic and the zone of inhibition in terms of physical constants.

$$\log m_0 = \frac{1}{4D\,2.30} \cdot \frac{x^2}{t} + \log m'$$

where

D = the coefficient of diffusion of the solute
x = the distance from the inner face of the cup to the edge of the zone of inhibition
m_0 = the concentration of the antibiotic in the cup
m' = the smallest concentration of antibiotic showing inhibition on a solid medium
t = the critical time of growth beyond which the test organism shows no perceptible inhibition

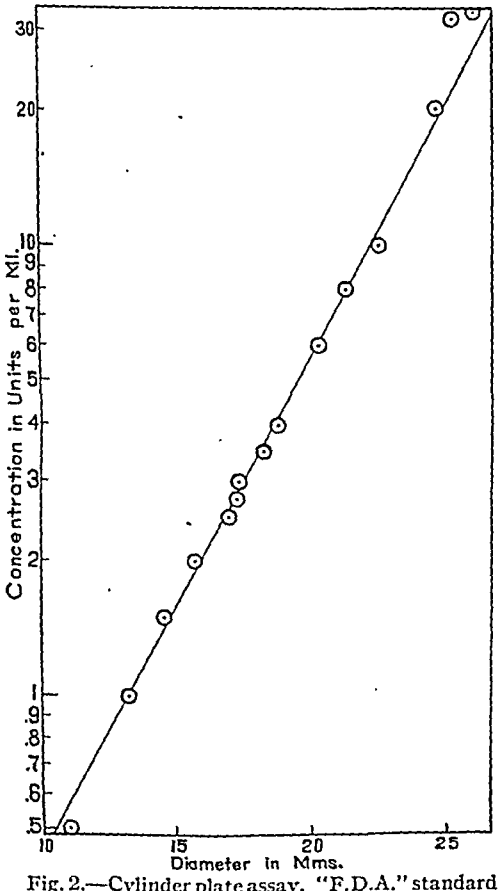

Fig. 2.—Cylinder plate assay. "F.D.A." standard curve. Zone diameters in mm. *versus* logarithms of bacitracin concentration.

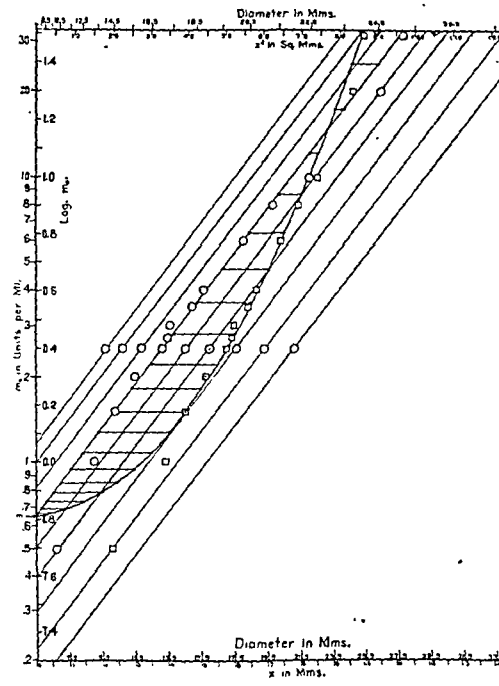

Fig. 3.—Cylinder plate assay. Cooper–Woodman standard curve. Log m_0 *versus* x^2. Circular symbols represent values of x^2. Values of log m_0 *versus* x (square symbols).

In Fig. 3, based also on Table I, the concentration of bacitracin is plotted on a logarithmic scale against values of x^2 on a linear scale. The resulting graph is shown by a straight line established by points which are designated by the circular symbols. On this curve fairly good correlation exists over a range of inhibition zones from 14.5 to 21.0 mm., which correspond to approximately 1.5 to 8 units per ml. Standard curves for other values of the 2.5 units per ml. standard have been indicated on this graph by lines parallel to the "17-mm." curve.

In order to calculate values directly from this graph it is only necessary to plot corresponding values of zone diameters from the "17-mm." curve on a suitable overlapping linear scale as shown in Fig. 3 by the curved line. Then from values of the zones of inhibition read on the lower scale one may read units directly on the logarithmic scale. It is of interest to note here that if values of x are plotted on the linear scale against log m_0, as has also been done in Fig. 3, a straight line relationship is again found to exist. In this instance the equation may be expressed as $\log m_0 = b'x + \log a'$.

Although the Cooper–Woodman formula is of theoretical rather than practical interest it has been used in our laboratory to calculate a chart for the assay of bacitracin. The work involved was cumbersome, however, and the method has no advantage over the simpler F. D. A. procedure.

The Coefficient of Diffusion.—The coefficient of diffusion of bacitracin was determined by the

Cooper–Woodman method from a standard curve of bacitracin against *S. aureus* without preliminary refrigeration. In this case the zones of inhibition were small and the curve was extremely steep. For example, when m_0 was 5.0 units per ml., x^2 was found to be 18.06 sq. mm. and m' was 0.90 unit per ml. The value of t was determined by incubating *S. aureus* plates with the unfilled cylinders in position. After different intervals of time the cylinders were filled with the 2.5 units per ml. standard. In this manner it was found that with an elapsed time of five hours before filling there was no inhibition of the test organism. By substitution of these values in the theoretical equation, the coefficient of diffusion, D, was found to be approximately 0.53 sq. mm per hour.

Cooper and Woodman have reported the coefficients of diffusion of crystal violet as 0.30 sq. mm. per hour and of penicillin as 1.06 to 1.57. The value 0.53 for bacitracin is therefore probably about one-half to one-third of that of penicillin. This difference in diffusion rates is in line with the pharmacological findings that bacitracin is not absorbed into the blood stream from the tissues as rapidly nor excreted as fast as penicillin. For example, significant concentrations of bacitracin persist in the blood stream as long as seven to eight hours after a single subcutaneous or intramuscular injection, while penicillin blood levels drop in two or three hours. Scudi (7) reports that urine levels of bacitracin may persist for at least 24 hours after a single injection while penicillin disappears in six to eight hours. Thus the cup plate method may permit some generalizations to be made on the movement of the antibiotic through the tissue cells. In this respect it is unwise for investigators searching for new antibiotics to discard all organisms which produce only a small zone of inhibition on agar plates.

SUMMARY

1. The antibiotic, bacitracin, can be assayed by essentially the same methods which are in general use for penicillin. A turbidimetric and a cylinder plate technique are described.

2. Methods for establishing standard curves and charts for the cylinder plate assay are described and the nature of the formula of the graph is discussed in detail.

3. The coefficient of diffusion calculated from the Cooper–Woodman formula was found to be about one-half to one-third of that of penicillin. This is in agreement with published pharmacological findings which show slower excretion rates for bacitracin than for penicillin.

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DISPENSATORY ERRATA ANNOUNCED

The attention of owners and users of the 24th Edition of the "United States Dispensatory" is directed to certain corrections of typographical errors in the first impression of the book.

Page 496—Line 15, column 2: The number 16 in this line should be changed to 6.

Page 794—The amount of antimony and potassium tartrate specified in the formula for Compound Opium and Glycerrhiza Mixture should be changed to read 0.24 Gm. instead of 24 Gm.

Page 1573—Line 2, column 2: Change "lucite" to "leucite."

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Ascaridol in Oil of Chenopodium*

By ALFRED HALPERN†

The methods proposed for the determination of ascaridol content of oil of chenopodium have been reviewed and the literature surveyed. The possibility of utilizing the oxidative property of ascaridol on ascorbic acid as an assay procedure has been investigated. There was no evidence of a stoichiometric relationship between the reactants. Emphasis is placed on the need for a re-investigation of the oxidative behavior of ascaridol because of the nonconformance among the various assays between the experimental and calculated values based on the theoretical explanation of these reactions.

OIL OF chenopodium has a relatively wide therapeutic use as a vermifuge. Smillie and Pessoa (1) have shown that ascaridol was the sole component of the oil which exhibits anthelmintic action against hookworm and round worm, for which the oil is generally used. A determination of the ascaridol content of the oil of chenopodium is therefore important from the therapeutic standpoint; also as oil of chenopodium commands a fairly high price, it is consequently further liable to adulteration. This was the case of the "synthetic" chenopodium oils to which attention was called by Schimmel and Company in their reports for 1919 (2).

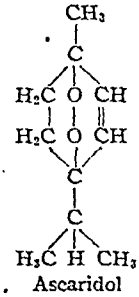
Ascaridol is unstable to heat and is liable to explode above 100°. It forms no known solid derivatives by which it can be characterized. It is capable of being reduced to the corresponding glycol by ferrous sulfate and to 1,4 terpin by hydrogen in the presence of palladium (3).

The U. S. P. X recognized the oil of chenopodium, but apart from physical constants there was no determination for ascaridol content.

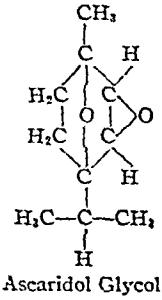
METHODS REVIEWED

Schimmel and Company (4) determined the ascaridol content of chenopodium oil by vacuum distillation to be 62–65% in the normal oil and 45–50% in the light oil. This method is slow and yields results obviously dependent upon the technique employed and which do not account for the ascaridol altered by the distillation process. Nelson (5) showed that ascaridol undergoes molecular rearrangement when heated to 150°. He further found that a bath temperature of 80° at 3–6 mm. pressure was sufficient for the terpene fraction. A bath temperature of 115° at 3–6 mm. pressure gave an even distillation of the ascaridol fraction. As a secondary check on the distillation method, Nelson employed a calculation of the ascaridol content of the oil by its density, assuming that the density of the terpenes was 0.8466 and that of ascaridol was 1.005. No other constituents were assumed present in the oil. The figures thus obtained run roughly parallel to those of the direct distillation. The limitations of this method are readily apparent and do not serve for an estimation of ascaridol content in an "unknown" oil.

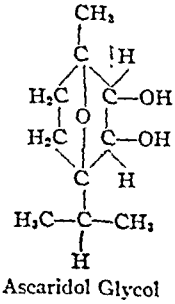
A rapid method of determination was suggested by Nelson (6) based on the solubility of the ascaridol portion in 60% acetic acid. The terpenes present in



Ascaridol



Ascaridol Glycol Anhydride



Ascaridol Glycol

* Received Sept. 29, 1947, from the College of Pharmacy, State University of Iowa, Iowa City, Iowa.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.
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the oil of chenopodium were shown to be insoluble in 60% acetic acid. The method is simply to agitate 10 cc. of the oil of chenopodium with 60% acetic acid in a cassia flask. The neck of this flask is to be graduated in tenths. The flask is then filled to the mark with 60% acetic acid and allowed to settle or is centrifuged. The ascaridol content was determined from the difference in height between the original and treated oil columns.

This method of assay has many deficiencies: first, this method does not distinguish between ascaridol and the intramolecularly rearranged products (ascaridol glycol anhydride and ascaridol glycol) which are soluble in 60% acetic acid. Cineol, a component of oil of chenopodium, is also soluble in 60% acetic acid. Second, from the standpoint of technique, the time of shaking, the period of standing, the advisability of centrifuging, and the method of reading the oil column are left to the individual worker. Third, no method is given for determining the ascaridol content in oils heavier than the specific gravity of the 60% acetic acid. Such would be the case if carbon tetrachloride, another common vermifuge, were present.

Paget (7), after pointing out some of the above deficiencies of the method, suggested that the oxidizing property of ascaridol be used to determine the concentration. He devised a method based on the reduction of titanous chloride ($TiCl_3$), stabilized with hydrochloric acid. This method has an empirical factor taken from the average of several experiments, and was based on the mean value of 1 Gm. of ascaridol, reduced by 1.277 Gm. of titanous chloride. The author does not claim a high degree of accuracy for the method. He further states that the specific gravity was a useful guide to the ascaridol content, and that the solubility in 60% acetic acid was the most rapid and convenient for field work. For detection of highly adulterated samples the reduction technique or the distillation method was best.

In a later paper Nelson (8) recognized the limitations of the acetic acid solubility method, then official in the U. S. P. X. A sample containing 50% ascaridol, 20% turpentine, and 30% eucalyptol assayed as 85% ascaridol by the acetic method and as 43.2% by the Paget method.

A report of the committee (9) established to investigate the method of assay of oil of chenopodium for ascaridol content, by the Association of Official Agricultural Chemists, compared both methods of analysis (*viz.*, Nelson's and Paget's). The empirical factor of Paget was established as 1.284 (a difference of 0.007 from the original factor) and was arrived at as the mean of sixteen determinations on a highly purified ascaridol fraction. The difference between their factor and that of Paget was attributed to the different concentrations of hydrochloric acid used to stabilize the titanous chloride in both studies.

When the acetic acid method and the Paget method were used to determine the ascaridol content on known samples, two sets of results were obtained (9). In the first, the Nelson assay gave higher values than the Paget method. In the second, the reverse was noted. The high result of the Nelson method, in the first case, was accounted for by the presence of *p*-cymene, the principal constituent of the hydrocarbon fraction of the oil of chenopodium. Cymene and 60% acetic acid are partly miscible. Ascaridol is miscible with cymene in all proportions. Therefore, if an oil of chenopodium containing cymene was mixed with 60% acetic acid, the ascaridol was distributed over the two layers. Thus two conjugate ternary systems were produced, each containing ascaridol, cymene, and acetic acid. These two systems were in equilibrium with the other and the composition of each would depend on the concentration of the three components and the temperature of the mixture. In the Nelson assay, the cymene would always contain some ascaridol, the amount dependent upon the concentration of cymene in the oil. Thus, less ascaridol was accounted for by this method of assay. This was confirmed by a series of dilution studies on a highly purified ascaridol and cymene mixture.

Knaffl-Lenz and Hoffman (10) devised a method based on the color developed between ascaridol and hydrochloric acid. To a 1% solution of the oil in alcohol was added 1 cc. of concentrated hydrochloric acid. The mixture was allowed to stand a number of hours, six being considered sufficient. (However, better results were obtained on standing twenty-four hours.) The samples were then compared in a colorimeter with a standard solution of 100% ascaridol treated in the same way. Accurate readings were hard to obtain because of the presence of dark insoluble oil droplets in every mixture. (It was found necessary to dilute each sample in order to obtain a sufficient quantity for use in the colorimeter.) Furthermore, terpineol, an adulterant studied, was found to give an added color to the solutions. A solution of 50% ascaridol in terpineol gave a value of 55%.

Other color reactions that take place when ascaridol-containing oils are treated physically and chemically have been suggested as a measure of ascaridol (11, 12). These methods are more of a qualitative nature and do not lend themselves to an exact quantitative determination.

Cocking and Hymas (13) introduced a method of analysis based upon the liberation of iodine from potassium iodide by the organic peroxide ascaridol. The method required that 3 cc. of potassium iodide solution (83% *w/v*) be mixed with 5 cc. of concentrated hydrochloric acid and 10 cc. of glacial acetic acid and cooled to −3°.

To this solution, after cooling, was added 5 cc. of the oil sample prepared by diluting 2.5 Gm. of the oil to 50 cc. with 90% acetic acid. The flask was stoppered and allowed to stand in a cool place for five minutes. It may be left for ten minutes at temperatures below 10°. The contents were then titrated with 0.1 *N* thiosulfate solution. A blank was carried out precisely as described except that the final solution was diluted with 10 cc. of water before

titrating. It was found necessary to employ an empirical factor, the relation being 1 cc. of 0.1 *N* thiosulfate equals 0.00665 Gm. of ascaridol.

This method has several limitations. The authors claimed that three reactions occurred in sequence. Two moles of iodine were liberated from the acidified potassium iodide solution, followed by an unexplained liberation of iodine and then a reabsorption of iodine when the solutions were diluted. This method, on the basis of limited determinations, was found not to be well suited to high-grade oils. The chances of error were great unless rigorous specifications were followed. This method was adopted by the U. S. P. XII and N. F. VIII.

In comparing the iodine method with the Paget method, the former has the advantage of not requiring the precautions necessary to stabilize the titanous chloride and also does not require a neutral atmosphere. However, from the point of view of time it is the same as that of Paget's method and it also employs an empirical factor.

"Broughton felt that the iodine assay was worthy of further study, in spite of the number of factors that influenced the accuracy. Dafert and Capesius found it simple and accurate. Bodendorff felt that the development of an exact quantitative method for ascaridol based on an iodometric procedure is highly improbable" (14).

In an attempt to devise a biological method, Reindollar and Munch (15) came to the conclusion that no relationship exists between the lethal dose and ascaridol content as determined chemically. Knaffl-Lenz and Hoffman (10) reported similar results.

In view of the above difficulties for the determination of ascaridol, it was decided to investigate the behavior of ascorbic acid in the presence of ascaridol and oil of chenopodium. The organic peroxide presumably should oxidize the sensitive ascorbic acid, and since ascorbic acid could be fairly easily determined by titration with dichlorphenolindophenol the quantity of ascaridol present in a sample would be indicated by the amount of ascorbic acid oxidized.

EXPERIMENTAL

An ascaridol fraction was obtained by distillation of oil of chenopodium at 85–95° at 2–5 mm. Hg. This fraction was redistilled twice and corresponded to 59% of the quantity of oil taken. The specific gravity of this fraction at 20° was 0.998 and when assayed by the Nelson method corresponded to 100% ascaridol and 99.8% by the potassium iodide method.

A preliminary series of reactions with ascaridol and ascorbic acid were run to ascertain the oxidative effect of ascaridol. A rather wide variation of values was obtained. It was thought that the uneven distribution of the oil in the water was responsible for the variations observed. Alcohol was substituted for a large part of the water and the stability of the ascorbic acid in this medium was determined.

To 0.01 mole of ascorbic acid dissolved in 150 cc. of water, was added 450 cc. of 95% alcohol. The solution was kept in an ice bath at 0° throughout the study. At the indicated intervals an aliquot portion was removed and titrated with standardized dye.[1] The amount of ascorbic acid remaining was referred to the original dilution (Fig. 1). The stability of the system was thus established.

Determinations on the purified ascaridol fraction obtained by distillation were attempted.

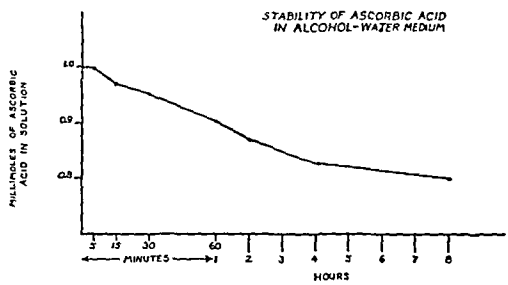

Figure 1

Method A.—One millimole of ascaridol in 15 cc. of 95% alcohol was mixed with a solution of 5 millimoles of ascorbic acid in 5 cc. of water. The mixture was cooled at 0°, in an ice bath, for ten minutes, with constant shaking. The mixture was diluted with 10 cc. of water and titrated with standardized dye. The amount of ascorbic acid oxidized by 1 millimole of ascaridol was thus determined (Table I). A blank was run without ascardiol.

TABLE I.—OXIDATION OF ASCORBIC ACID BY 1 mM OF ASCARIDOL[a]

Sample No.	mM of Ascorbic Acid Oxidized
1	1.6
2	1.4
3	1.8
4	1.6
5	2.0
6	1.4
	Av. 1.6 ± 4% A.D.

[a] Empirical factor = 1 mole of ascaridol oxides. 1.6 moles of ascorbic acid.

The determination was repeated at increased time intervals to determine whether the value obtained was influenced by the length of time the ascaridol was in contact with the ascorbic acid. The determination was carried out in the described manner except for the time interval (Table II). The length of time the ascorbic acid remained in contact with the ascaridol at 0° apparently did not influence its oxidation within certain limits. There was an appreciable increase in the oxidation of ascorbic acid at room temperature (24°). The time limit was arbitrarily maintained at ten minutes at 0°.

[1] Sodium Dichlorphenolindophenol, standardized by procedure given in the U. S. P. XIII, p. 620.

TABLE II.—INFLUENCE OF TIME ON THE OXIDATION OF ASCORBIC ACID BY ASCARIDOL

Sample No.	Time, Min.	Temp., °C.	mM of Ascorbic Acid Oxidized
1	5	0	1.4
2	10	0	1.8
3	15	0	2.0
4	20	0	1.7
5	25	0	1.4
6	30	0	2.1
7	(5 at (plus 5 at	0) 24)	2.3
8	5	24	2.6
9	10	24	2.8

Method B.—Following the above procedure for distilled ascaridol approximately 0.5 Gm. of oil of chenopodium was dissolved in 15 cc. of alcohol and mixed with 5 cc. of a solution of 5 millimoles of ascorbic acid in distilled water. The mixture was cooled in an ice bath to 0° and agitated for ten minutes. At the end of this time the mixture was diluted with 10 cc. of water and titrated with standardized dye solution (Table III).

TABLE III.—DETERMINATION OF ASCARIDOL BY ASCORBIC ACID METHOD

Sample No.	Sample Weight	mM of Ascorbic Acid Oxidized[a]	Equiv. mM of Ascaridol[b]	Ascaridol, Gm.	Ascaridol, %
1	0.4263	2.9	1.8	0.3027	71
2	0.3214	2.6	1.6	0.2764	86
3	0.4210	2.2	1.4	0.3283	78
4	0.2170	1.8	1.1	0.1802	83
5	0.4983	3.34	2.1	0.3587	72

[a] Corrected with blank determination run simultaneously with experimental.
[b] Based on relationship derived from Table I.

This oil of chenopodium was then assayed by the acetic acid method (Nelson) and was found to contain 68.5% of ascaridol. This value was checked by the iodine method and was found to contain 66.8% of ascaridol.

The abnormally high results obtained from the determinations made on the oil of chenopodium indicated that there was some additional factor present in the oil that oxidized the ascorbic acid. An attempt was made to determine the activity of the fractions obtained by distilling the oil under reduced pressure. The fraction distilling at temperatures up to 80° under 3–5 mm. pressure was separated. On assay by the iodine method, this fraction was found to contain 6% of ascaridol. This fraction was further purified by washing with 60% acetic acid and then neutralized by washing with 5% sodium bicarbonate solution. On assay of this sample no ascaridol was found by either the iodine method or the acetic acid method. This fraction was then assayed with ascorbic acid in the manner described above. Approximately 0.5 Gm. of this sample was used (Table IV).

DISCUSSION

The oxidation of ascorbic acid by ascaridol did not proceed in the expected mole-mole relationship. There was some extraneous factor present in this system that was also reacting with the ascorbic acid. Since we sought a means of determining the ascaridol content of oil of chenopodium that would be applicable to the usual laboratory equipment, specially prepared apparatus and reagents were not used. Rather, the routine glassware found in any well-equipped laboratory was cleaned in the usual manner, rinsed with distilled water, and air-dried before use. The distilled water was delivered from a block tin system and met the U. S. P. XII requirements. It is therefore necessary to point out the sensitivity of ascorbic acid to trace concentrations of metals. While it was felt that the influence of such a variable was kept at a minimum, as evidenced by the stability study of the system and in the blank determinations run simultaneously with the assay, this factor must be considered in a valid discussion of ascorbic acid oxidation.

The nonconcurrence among the values obtained as a result of the determinations made on the distilled ascaridol indicated a behavior of ascaridol that was not quite understood. It is of interest to note that none of the oxidation-reduction reactions thus far investigated for assay purposes afforded results that agreed stoichiometrically. The consistent failure among the various assays to obtain values that correspond to the theoretical, based on a mole-mole relationship, necessitates a re-examination of the mechanism and resulting products from these reactions. The reproducibility of the empirical values obtained from the iodine and titanous chloride assays indicates that these reactions behave in a systematic manner and that a factor, as yet unexplained, was the determinant in these reactions.

The evidence of some oxidation of ascorbic acid by the terpene fraction of oil of chenopodium was rather interesting. It was impossible to state on the basis of these limited results whether the latent oxidation was due to one of the constituents of this fraction (i.e., *p*-cymene) or to some rearranged product of ascaridol which resulted from the distillation procedure. However, the values obtained from these experiments (Table IV) do not account

TABLE IV.—OXIDATION OF ASCORBIC ACID BY TERPENE FRACTION

Sample No.	Sample Weight	mM of Ascorbic Acid Oxidized[a]	Equiv. mM Ascaridol[b]	Ascaridol, Gm.	Ascaridol, %
1	0.4215	0.32	0.20	0.0337	8
2	0.5126	0.50	0.31	0.0517	10
3	0.4613	0.80	0.50	0.0846	12
4	0.4461	0.21	0.13	0.0223	5
5	0.3314	0.23	0.14	0.0232	7

[a] Corrected with blank determination run simultaneously with experimental.
[b] Based on relationship derived from Table I.

for the difference in ascaridol content as determined by this method and the others on the same oil.

The extreme variation among the values obtained on the distilled ascaridol and also among the different samples of the same oil of chenopodium invalidated further investigation with other preparations from different sources.

The reagents used to determine the active ingredient of a drug should evidence a measure of specificity for that agent. The results of this investigation indicate no such specificity. The application of the above method to the determination of ascaridol in oil of chenopodium must be viewed negatively for the above reasons.

SUMMARY

1. The methods proposed for the determination of ascaridol content of oil of chenopodium have been reviewed and the literature surveyed.

2. The possibility of utilizing the oxidative property of ascaridol on ascorbic acid as an assay procedure has been investigated.

3. There was no evidence of a stoichiometric relationship between the reactants. The wide variation of values that was obtained with distilled ascaridol and oil of chenopodium invalidates this method as an analytical procedure.

4. Emphasis is placed on the need for a re-investigation of the oxidative behavior of ascaridol because of the nonconformance among the various assays between the experimental and calculated values based on the theoretical explanation of these reactions. All described methods for the determination of ascaridol content employ an empirical factor. The results of the various assays are reproducible indicating a systematic constant order of reaction that is not explained by the theoretical considerations proposed for the reaction.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Montanic acid
Glucoascorbic acid
Acenaphthylene
Cuscohygrine
Hemipinic acid
Hordenine
4-Hydroxyquinoline-3-carboxylic acid
4-Phenyl-2-aminobutyric acid
Undecyl aldehyde
Scopoletin
N-Methylnipecotic acid
Ethyl vanillate
Thiocytosine
Phosphatase, purified
Hydroxylamine-O-acetic acid hydrochloride
Methoxinine
d-Erythrose
d-Threose
17-Hydroxycorticosterone
11-Dehydro-17-hydroxycorticosterone
Stigmasterol
Maltobionic acid

A Quantitative Microscopic Study of Certain Commercial and Vegetable Drug Starches*

By ROBERTS L. PROPER† and H. W. YOUNGKEN, JR.‡

The existing methods employed for starch counting in starch mixtures have been studied and an improved method based upon the use of a saturated solution of zinc sulfate and a Levy blood counting slide is proposed. The selection of a suspending agent of close proximity to the specific density of starch, the latter being at the same time not altered, provided for easier handling of the sample, rapid counting, and more consistent results. Methods are described for extracting pure starch from the vegetable drugs belladonna, aconite, glycyrrhiza, and orris roots. Limitations and disadvantages of the proposed method are described.

THE occurrence of starch granules in cereal products and vegetable drugs is of prime importance to the microscopist who endeavors to identify such substances in certain mixtures. The microscopic identification of starch granules has as its basis a knowledge of the structural characteristics of each type of starch. Along with these morphological characteristics, the microscopist frequently has need to determine the amount of starch present in a given mixture by means of quantitative methods. The most acceptable methods for the quantitative determination of starch are based upon the enzymatic and chemical hydrolysis of the starch substance with subsequent formation of sugars and reduction of a copper-containing solution.

Quantitative methods for the determination of starch, other than the chemical methods, are recorded in the literature: for example those of Meyer (1, 2), Hartwich and Wichmann (3), Hart (4), Allen (5), Chamot (6), and Wallis (7). Schneider (8) and, more recently, Mendelsohn (9) have applied counting methods with some degree of success to the analysis of starch in baking powder mixtures and the microanalysis of vegetable drugs. In general, these methods involve the weighing of the starch mixture with subsequent suspension of the starch granules in a suspending medium and a microscopic counting of the starch by means of ruled slides. Such methods have generally fallen into disuse, due largely to certain technical difficulties such as occur during the handling of samples before counts are made in prescribed counting chambers and failures to establish a satisfactory comparison with the established chemical methods.

The problem forming the subject for this investigation arose following a desire to investigate the existing nonchemical quantitative methods of starch analysis and, where applicable, to devise a method which would give results more comparable with those from the accepted chemical methods.

MATERIALS AND METHODS

For the first phase of this study, the following commercial starches were used: corn, potato, wheat, and rice. Each starch was examined microscopically for the presence of altered granules, dextrins, fibers, and foreign substances.

Purified Talc and Purified Siliceous Earth were used in making unknown starch mixtures.

Starches were extracted from authentic fresh samples of four different vegetable drugs. These starches were used for the determination of reference standard starch counts for each drug studied. Such material was prepared from fresh root portions of Belladonna (*Atropa belladonna* L.), Glycyrrhiza (*Glycyrrhiza glabra* L.), Iris (*Iris pallida* Lamarck), and Aconite (*Aconitum napellus* L.).

The preparation from the fresh root portions of each plant was performed in the following manner.

The fresh materials were thoroughly washed and then peeled. The peeled drug was soaked in warm water (50°), containing 0.05% sulfurous acid (approx. 6%) until the drug material was thoroughly saturated. The sulfurous acid acted as a bleaching agent and removed most of any remaining pigments or tannins present in adhering rind. The material was again washed and then placed in a Waring

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Blendor and thoroughly disintegrated into a pulpy mass. Enough water was added to facilitate removal of the pulpy mass from the Blendor. The milky mixture was then passed through a #60, #100, and #200 sieve in that order. The residue present on each sieve was washed until all starch had been removed. The milky starch-bearing liquid from the #200 sieve was placed in a large aspirator bottle and allowed to flow at the rate of approximately 30 drops per minute on a metal settling and washing trough. The trough was 5 feet long and fitted with baffle boards running transversely every 6 inches of its length. After all the starch-bearing water from the aspirator had passed down the trough, the residue (starch) remaining on the trough was washed slowly with pure water. This was done until, upon microscopic examination, only pure starch remained in the trough. The starch residue was removed from the trough with a small amount of water and strained through four thicknesses of fine grade silk bolting cloth. The residue was washed with water and the strained liquids were filtered by means of suction through a smooth hard filter paper (Whatman's No. 50). The starch was then dried by means of a small amount of 95% alcohol and then a small amount of ether. Finally the starch was removed from the filter and air dried in an evaporating dish.

Authentic samples of powdered vegetable drugs were used as unknowns in determinations involving vegetable drug starches. These samples were examined microscopically for adulteration and for alteration of starch granules. A hemocytometer slide was used as the counting chamber for the microquantitative procedures. This slide consisted of a Levy counting chamber with the improved Neubauer ruling.

The methods used for the determination of starch quantitatively by means of chemical analysis were those outlined in "Methods of Analysis of the Association of Official Agricultural Chemists" (10). The procedure was followed as described except that in place of malt diastase a saturated solution of Takadiastase was used.

EXPERIMENTAL

Part I. The Examination of Commercial Starches

Several samples of the common commercial starches of corn, wheat, potato and rice were examined to verify their authenticity. They were then mixed with common suspending agents, such as aqueous solution of acacia or mucilage of tragacanth. These were used in concentrations of 5%, 15%, 25%, and 50% solutions. Glycerin and water mixtures were used in concentrations of 10%, 25%, 50%, and 100% of glycerin. A chemically pure grade of triethanolamine was also used as a suspending medium. These agents were selected because of the use of each in the different starch counting methods as reported in the literature (2, 3, 6, 8, 9). The advantages and disadvantages of each medium were studied and found to be as follows: The vegetable gums in concentrations of 25% and 50% appeared to give the best suspension after considerable trituration and shaking. However, upon examination, the starch granules were observed to have a tendency to aggregate into small clumps. In the lower concentrations, the starch was not suspended satisfactorily for counting purposes. The glycerin and water mixtures and the triethanolamine were also found unsatisfactory for use in counting starch microscopically. The difficulty in obtaining a sufficiently homogeneous suspension for starch counting with the use of these suspending media was attributed in part to the fact that all suspending agents possessed too high a viscosity for satisfactory mixing with starches. On this assumption it was decided that a suspending medium having a viscosity lower than that of the above media and with a specific gravity as near that of starch as would be possible might produce a more homogeneous suspension. With an agent of this type, the suspension could then be made by placing the weighed sample directly into the medium, and shaking vigorously. The trituration necessary with the other suspending agents could thus be eliminated. In addition, the use of a more efficient suspending medium of this type would eliminate the need for making a starch paste in one receptacle and pouring the resultant mixture into another. It was noted that errors in starch counting were caused by both of these procedures. In this case the efficiency of the suspension would depend more upon the medium's specific gravity than upon its viscosity. Of those substances which have a specific gravity comparable to that of starch, concentrated solutions of certain inorganic salts appeared to be the most acceptable.

Among the inorganic salts used were potassium iodide, copper sulfate, copper nitrate, potassium carbonate, lead acetate, zinc chloride, and zinc sulfate. Of these salts a saturated solution of zinc sulfate was found to be the most suitable. The other salts either caused a rupturing and alteration of the starch granules or had a specific gravity that was too low for producing an efficient suspension of the starch.

Starch granules even after standing overnight in saturated solution of zinc sulfate showed no swelling or alteration effects of any kind. This agent was found to have a specific gravity almost exactly that of starch, *viz.*, approx. 1.5. A necessary precaution when using this solution was that to insure thorough suspension there must be vigorous shaking of the starch-zinc sulfate mixture.

A Levy blood counting slide was employed for making counts because of its standardization simplicity and ease in handling. The large corner squares were ideal for counting the larger starch granules, such as potato starch; the smaller central squares were used for the smaller starch granules, such as corn, wheat, and rice.

In order to ascertain the efficiency of a counting system it was necessary, first, to determine a stand-

ard count for a definite volume of a known dilution of the pure starch suspended in the zinc sulfate solution. This procedure was carried out in the following manner. A convenient amount of the pure starch, depending on the size and number of the granules present, was accurately weighed. These weights were from 20 mg. to 100 mg. The sample was then carefully transferred to a 10-cc. volumetric flask, and a few cubic centimeters of the saturated zinc sulfate solution added by means of a pipette. The flask was then shaken vigorously for two or three minutes and three drops of iodine test solution added. The flask was again shaken until the iodine was completely mixed and then zinc sulfate solution added to the 10-cc. mark. After thorough shaking a drop of the suspension was placed on the counting area of the Levy blood counting slide by means of a small capillary pipette. This pipette consisted of a piece of glass tubing drawn out into a tube of capillary dimensions. The first drop from the pipette was discarded and the second drop placed directly upon the counting area. The cover slip was then carefully laid in place taking care that no air bubbles were present and that none of the suspension had run over into the wells surrounding the counting area. If either of these errors occurred precautions were taken to clean the slide thoroughly, and to reload it with another drop of suspension from the capillary pipette.

In order to attain a standard figure for a given starch with any degree of accuracy it was deemed necessary to make at least eight separate counts. This necessitated loading and cleaning the slide and cover slip eight separate times. In this procedure there were certain precautions to be observed. These consisted of shaking the flask in order to resuspend any of the starch that had settled to the bottom and any suspension remaining in the pipette was blown back into the flask. Following this the pipette was wiped off on the outside and then inserted into the flask. The pipette was allowed to fill by capillary action and not by the usual method of applying suction.

After the eight counts were made the results were added and an average taken. These results were obtained when using the large squares by counting the starch granules in each square. The figures thus obtained were then added and divided by four to give the number of starch granules per 0.1 cu. mm. If the smaller central squares were used the total count of five was made and then multiplied by five thereby attaining the number of starch granules per 0.1 cu. mm. The figure obtained from either of these procedures was then accepted as being the standard for the specific starch and was used on all other determinations of that starch or mixtures containing it.

As an example the determination of the standard reference count for potato starch was performed in the following manner. The starch after having been suspended and obtained as previously described was placed on the hemocytometer and counted in the four large corner squares. The total number of starch granules in the four squares was then divided by four in order to obtain the number of granules in 0.1 cu. mm. This procedure was followed eight times and the average of the eight counts was found to be 147 granules per 0.1 cu. mm. This same procedure was followed in the case of starches with smaller granules except that five of the small central squares were counted, totaled and multiplied by five to obtain the standard count for the specific starch.

TABLE I.—STANDARD MICROQUANTITATIVE STARCH COUNTS FOR COMMERCIAL STARCHES

Name of Starch	Weight/10 Cc. of Suspension,[a] Mg.	Type Square of Counting Chamber Used	No of Counts Made	Record of Individual Counts Made	Av./0 1 Cu. Mm.
Corn	100	Small	8	1900, 1725, 1800, 1725, 1950, 1925, 2000, 1775	1850
Wheat	50	Small	8	475, 460, 465, 485, 540, 530, 480, 520	494
Rice	50	Small	8	2390, 2310, 2175, 2185, 2250, 2240, 2195, 2375	2265
Potato	100	Large	8	150, 149, 144, 146, 127, 133, 147, 179	147

[a] Starch counts were compiled with the use of a Levy blood counting slide with the improved Neubauer ruling. Saturated solution of zinc sulfate was used as the suspending medium.

Table I shows the results of determinations made in an effort to obtain standard starch counts for pure samples of each of four commercial starches, *viz.*, corn, wheat, rice, and potato. The average obtained for each of these starches as shown in Table I was used as the standard count for that specific starch. In order to determine the efficiency of the above starch-counting method and to check the accuracy of the standard counts mixtures containing known percentages of these starches were made. The same procedure was carried out and the standard count was used in computing the per cent starch present in the mixture. For example if 100 mm. of a mixture of potato starch and Talc were weighed and suspended in zinc sulfate solution and

the average of eight counts was found to be 74 starch granules per 0.1 cu. mm., the mixture would contain 74/147 × 100 = 50.34% potato starch.

The mixtures used in these determinations were made with either purified talc or purified siliceous earth as the diluent. In the case of wheat starch a sample of whole wheat flour was used as the unknown. Figures 1 and 2 show the appearance of potato starch and corn starch in these mixtures as they appear on the counting slide under the microscope. In each case the weight of the sample used in making the count was the same weight as that used in determining the reference count for the specific starch in question.

In addition to making starch counts of these mixtures a chemical assay was performed on the same sample. This procedure served as a check on the relative accuracy of the count system. At the same time it was used as a basis for comparing the results obtained by use of both methods. The results of these determinations are shown in Table II.

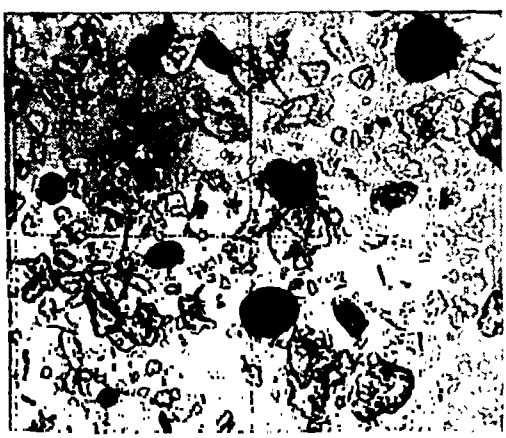

Fig. 1.—A mixture of Potato Starch in Purified Talc showing the starch granules to be counted in the large squares of the Levy blood counting chamber.

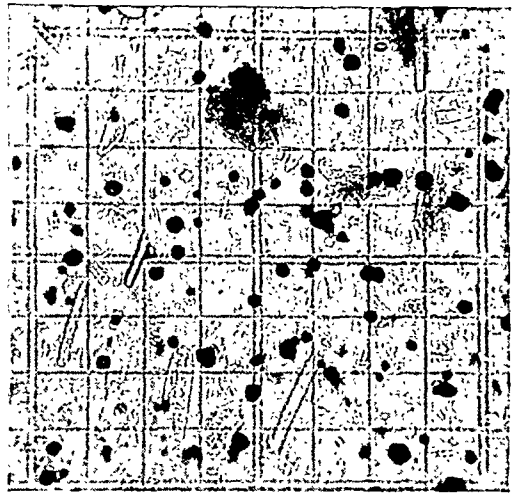

Fig. 2.—A mixture of Corn Starch in Purified Siliceous Earth showing the starch granules to be counted in the small squares of the Levy blood counting chamber.

These results show that in most cases the counting method and the chemical method compared favorably with one another and with the amount of starch that was actually present in the mixture.

Table III shows the results obtained in an attempt to determine the amount of potato starch in a known mixture of this starch with whole wheat flour. This determination indicated 0.54% more potato starch than the amount actually added to the whole wheat flour. However, the result is thought to be within the limits of experimental error expected from microscopic or chemical procedures. It was found difficult to differentiate completely between small potato starch granules and many wheat starch granules of the same size. This difficulty resulted in more time being required to complete the method than normally required with single starch mixtures. It was felt that a starch-counting pro-

TABLE II.—MICROQUANTITATIVE AND CHEMICAL STARCH ANALYSES OF COMMERCIAL STARCHES

Type of Starch	Diluent	Actual Starch, %	No. of Counts	Starch from Individual Counts, %	Av. by Counting, %	From Chemical Analysis, %	Av. of Chemical Analysis
Potato	Talc	10	8	10.03, 13.09, 8.33, 10.70, 9.40, 10.20, 9.40, 9.50	10.08	11.08 10.48 10.46 11.21	10.80
Corn	Siliceous Earth	25	8	27.83, 21.35, 26.75, 21.89, 24.59, 23.51, 27.59, 25.94	24.93	23.61 24.22 24.65 22.48	23.74
Wheat	Whole wheat flour		8	47.57, 48.58, 53.64, 52.63, 57.69, 51.61, 49.71, 53.47	51.86	52.72 49.16 51.27 51.05	51.03
Rice	Siliceous Earth	10	8	11.70, 11.20, 11.60, 10.90, 11.50, 11.00, 11.40, 11.50	11.30	10.92 8.95 10.26	9.61 8.95

ard count for a definite volume of a known dilution of the pure starch suspended in the zinc sulfate solution. This procedure was carried out in the following manner. A convenient amount of the pure starch, depending on the size and number of the granules present, was accurately weighed. These weights were from 20 mg. to 100 mg. The sample was then carefully transferred to a 10-cc. volumetric flask, and a few cubic centimeters of the saturated zinc sulfate solution added by means of a pipette. The flask was then shaken vigorously for two or three minutes and three drops of iodine test solution added. The flask was again shaken until the iodine was completely mixed and then zinc sulfate solution added to the 10-cc. mark. After thorough shaking a drop of the suspension was placed on the counting area of the Levy blood counting slide by means of a small capillary pipette. This pipette consisted of a piece of glass tubing drawn out into a tube of capillary dimensions. The first drop from the pipette was discarded and the second drop placed directly upon the counting area. The cover slip was then carefully laid in place taking care that no air bubbles were present and that none of the suspension had run over into the wells surrounding the counting area. If either of these errors occurred precautions were taken to clean the slide thoroughly, and to reload it with another drop of suspension from the capillary pipette.

In order to attain a standard figure for a given starch with any degree of accuracy it was deemed necessary to make at least eight separate counts. This necessitated loading and cleaning the slide and cover slip eight separate times. In this procedure there were certain precautions to be observed. These consisted of shaking the flask in order to resuspend any of the starch that had settled to the bottom and any suspension remaining in the pipette was blown back into the flask. Following this the pipette was wiped off on the outside and then inserted into the flask. The pipette was allowed to fill by capillary action and not by the usual method of applying suction.

After the eight counts were made the results were added and an average taken. These results were ol tained when using the large squares by counting th starch granules in each square. The figures thu obtained were then added and divided by four t give the number of starch granules per 0.1 cu. mm If the smaller central squares were used the tota count of five was made and then multiplied by fiv thereby attaining the number of starch granule per 0.1 cu. mm. The figure obtained from eithe of these procedures was then accepted as being th standard for the specific starch and was used on al other determinations of that starch or mixture containing it.

As an example the determination of the standar reference count for potato starch was performed i the following manner. The starch after havin been suspended and obtained as previously de scribed was placed on the hemocytometer an counted in the four large corner squares. The tota number of starch granules in the four squares wa then divided by four in order to obtain the numbe of granules in 0.1 cu. mm. This procedure was fol lowed eight times and the average of the eigh counts was found to be 147 granules per 0.1 cu. mm This same procedure was followed in the case o starches with smaller granules except that five of th small central squares were counted, totaled an multiplied by five to obtain the standard count fo the specific starch.

TABLE I.—STANDARD MICROQUANTITATIVE STARCH COUNTS FOR COMMERCIAL STARCHES

Name of Starch	Weight/10 Cc of Suspension,[a] Mg	Type Square of Counting Chamber Used	No of Counts Made	Record of Individual Counts Made	Av./0.1 Cu. Mm.
Corn	100	Small	8	1900, 1725, 1800, 1725, 1950, 1925, 2000, 1775	1850
Wheat	50	Small	8	475, 460, 465, 485, 540, 530, 480, 520	494
Rice	50	Small	8	2390, 2310, 2175, 2185, 2250, 2240, 2195, 2375	2265
Potato	100	Large	8	150, 149, 144, 146, 127, 133, 147, 179	147

[a] Starch counts were compiled with the use of a Levy blood counting slide with the improved Neubauer ruling. Satu rated solution of zinc sulfate was used as the suspending medium.

Table I shows the results of determinations mad in an effort to obtain standard starch counts fo pure samples of each of four commercial starches *viz.*, corn, wheat, rice, and potato. The averag obtained for each of these starches as shown i Table I was used as the standard count for that specific starch. In order to determine the efficiency of the above starch-counting method and to check the accuracy of the standard counts mixtures containing known percentages of these starches were made. The same procedure was carried out and the standard count was used in computing the per cent starch present in the mixture. For example if 100 mm. of a mixture of potato starch and Talc were weighed and suspended in zinc sulfate solution and

the average of eight counts was found to be 74 starch granules per 0.1 cu. mm., the mixture would contain 74/147 × 100 = 50.34% potato starch.

The mixtures used in these determinations were made with either purified talc or purified siliceous earth as the diluent. In the case of wheat starch a sample of whole wheat flour was used as the unknown. Figures 1 and 2 show the appearance of potato starch and corn starch in these mixtures as they appear on the counting slide under the microscope. In each case the weight of the sample used in making the count was the same weight as that used in determining the reference count for the specific starch in question.

In addition to making starch counts of these mixtures a chemical assay was performed on the same sample. This procedure served as a check on the relative accuracy of the count system. At the same time it was used as a basis for comparing the results obtained by use of both methods. The results of these determinations are shown in Table II.

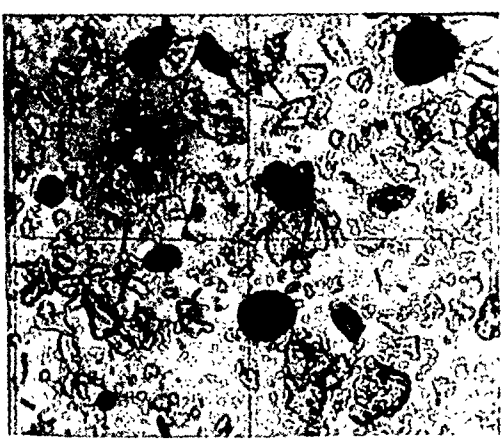

Fig. 1.—A mixture of Potato Starch in Purified Talc showing the starch granules to be counted in the large squares of the Levy blood counting chamber.

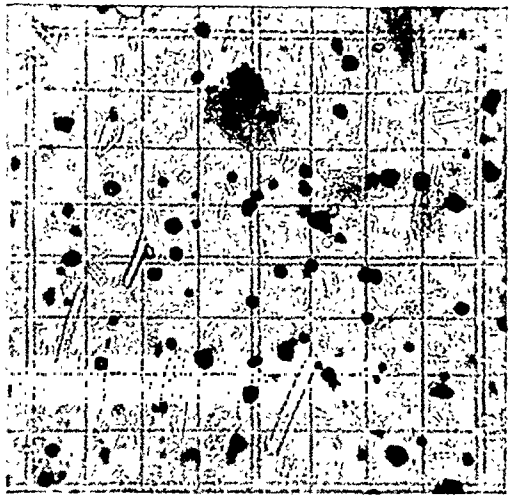

Fig. 2.—A mixture of Corn Starch in Purified Siliceous Earth showing the starch granules to be counted in the small squares of the Levy blood counting chamber.

These results show that in most cases the counting method and the chemical method compared favorably with one another and with the amount of starch that was actually present in the mixture.

Table III shows the results obtained in an attempt to determine the amount of potato starch in a known mixture of this starch with whole wheat flour. This determination indicated 0.54% more potato starch than the amount actually added to the whole wheat flour. However, the result is thought to be within the limits of experimental error expected from microscopic or chemical procedures. It was found difficult to differentiate completely between small potato starch granules and many wheat starch granules of the same size. This difficulty resulted in more time being required to complete the method than normally required with single starch mixtures. It was felt that a starch-counting pro-

TABLE II.—MICROQUANTITATIVE AND CHEMICAL STARCH ANALYSES OF COMMERCIAL STARCHES

Type of Starch	Diluent	Actual Starch, %	No. of Counts	Starch from Individual Counts, %	Av. by Counting, %	From Chemical Analysis, %	Av. of Chemical Analysis
Potato	Talc	10	8	10.03, 13.09, 8.33, 10.70, 9.40, 10.20, 9.40, 9.50	10.08	11.08 10.48 10.46 11.21	10.80
Corn	Siliceous Earth	25	8	27.83, 21.35, 26.75, 21.89, 24.59, 23.51, 27.59, 25.94	24.93	23.61 24.22 24.65 22.48	23.74
Wheat	Whole wheat flour		8	47.57, 48.58, 53.64, 52.63, 57.69, 51.61, 49.71, 53.47	51.86	52.72 49.16 51.27 51.05	51.03
Rice	Siliceous Earth	10	8	11.70, 11.20, 11.60, 10.90, 11.50, 11.00, 11.40, 11.50	11.30	10.92 8.95 10.26	9.61 8.95

cedure using mixtures of two or more starches would be applicable only in cases wherein starch granules of the same size could be accurately differentiated by means of definite morphological tests not investigated further.

TABLE III.—MICROQUANTITATIVE STARCH COUNTS —WHEAT FLOUR MIXTURE

Potato Starch Present in Whole Wheat Flour, %	Whole Wheat Flour Present, %	No. of Counts Made	Potato Starch from Individual Counts, %	Av. by Counting, %
10	90	8	7.4, 12.4, 9.1, 12.5, 9.7, 13.2, 9.52, 10.5	10.51

Part II. The Examination of Vegetable Drug Starches

The micro-quantitative starch counting method was applied to the examination of several powdered vegetable drugs in much the same manner as with the commercial starches. The drugs used in these determinations were Powdered Belladonna Root U. S. P., Glycyrrhiza U. S. P., Aconite N. F., and Orris Root N. F. Prior to counting the starch granules in these drugs it was necessary to obtain the pure vegetable drug starch in order to determine the standard starch count for each starch. These starches were obtained from fresh material by means of the washing process previously described. It was found that the fresh underground root portions of these plants were more satisfactory for use during this procedure than were the dried commercial drug materials.

Each starch from fresh drug sources was carefully examined for purity and alteration. Then the starches were weighed, suspended in the saturated solution of zinc sulfate, and counted in the same manner as were the commercial starches. The results obtained in determining the standard counts for these four vegetable drug starches are shown in Table IV. These results were used to calculate the

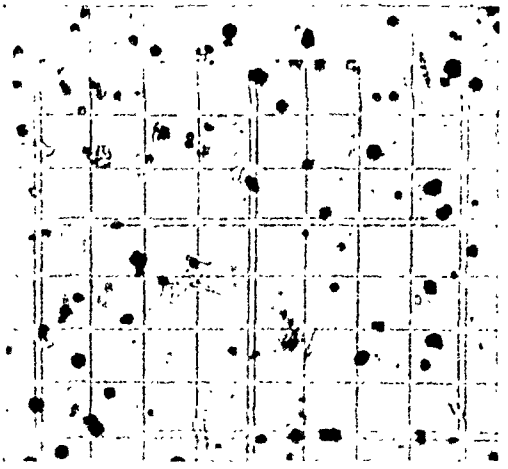

Fig. 3.—Powdered Belladonna Root showing the starch granules to be counted in the small squares of the Levy blood counting chamber.

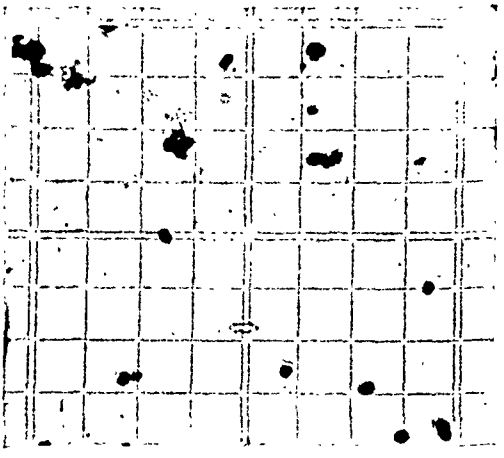

Fig. 4.—Powdered Orris Root showing the starch granules to be counted in the small squares of the Levy blood counting chamber.

TABLE IV.—STANDARD MICROQUANTITATIVE STARCH COUNTS FOR VEGETABLE DRUG POWDERS

Name of Starch	Weight/ 10 Cc. of Suspension,[a] Mg.	Squares of Levy Counting Slide Used	No. of Counts Made	Individual Counts	Av./0.1 Cu. Mm.
Belladonna	50	Small	8	1915, 1865, 2170, 2000, 2095, 2005, 1970, 2035	2007
Glycyrrhiza	20	Small	8	2365, 2495, 2415, 2510, 2100, 2165, 2580, 2430	2382
Aconite	25	Small	8	1220, 1160, 1340, 1400, 1165, 1180, 1165, 1220	1231
Iris (orris)	25	Small	8	470, 490, 520, 530, 655, 465, 650, 595	547

[a] A saturated solution of zinc sulfate was used as the suspending medium.

results of all subsequent determinations of each powdered drug containing that starch.

Following this, samples of the commercial powdered vegetable drugs, belladonna, glycyrrhiza, aconite, and orris roots, were counted and chemically assayed in order to determine the amount of starch present in each drug. The results of these determinations, both by the counting method and by the chemical method, are shown in Table V. Figures 3 and 4 show powdered belladonna root and powdered orris root as they appeared on the counting slide.

DISCUSSION

As a result of these determinations of commercial and vegetable drug starches, certain difficulties and precautions arise. Great care must be exercised when loading the counting chamber to insure that the starch granules are dispensed evenly. Occasionally some of the suspension on the outside of the pipette will drop on the slide causing an uneven dispersion of the starch granules. This can be remedied by wiping off the outside of the pipette with a clean towel before filling the counting chamber.

TABLE V.—MICROQUANTITATIVE AND CHEMICAL STARCH ANALYSES OF VEGETABLE DRUG STARCHES

Drug Used	Weight of Sample per 10 Cc. of Suspension,[a] Mg.	No. of Counts	Individual Counts, Starch, %	Av. Counting, Starch, %	% from Chemical Analysis	Av. of Chemical Analysis, %
Belladonna	50	8	21.67, 30.17, 32.38, 26.90, 29.90, 27.90, 26.15, 27.40	27.80	28.75 27.32 28.75 28.40	28.30
Aconite	25	8	21.93, 23.55, 23.15, 24.77, 22.64, 21.43, 21.65, 24.16	22.91	20.09 17.92 18.22 19.91	19.04
Glycyrrhiza	20	8	16.75, 12.80, 14.69, 14.69, 15.95, 16.75, 16.16, 16.79	15.56	13.29 13.12 14.05 13.34	
Iris (orris)	25	8	30.17, 31.09, 43.91, 53.83, 30.17, 48.48, 45.73, 46.65	38.75	39.52 40.05 37.63 39.08	

[a] Starch counts were made with a Levy blood counting slide. A saturated solution of zinc sulfate was used as the suspending medium.

As a result of the counting procedure it was found that the eight individual counts for each drug were variable but that the average of all eight counts compared closely with similar averages obtained from the use of the chemical procedure. Glycyrrhiza and aconite gave results which were considered to be somewhat higher than those obtained by the chemical method. It appeared significant that during the starch-counting procedure, using rice starch, the latter starch likewise showed a slightly higher count than noted following the chemical analysis method. Glycyrrhiza, aconite, and rice starches are examples of starch granules of small size, mostly 5 to 10 microns in diameter. The slightly higher results obtained when counting these starches were thought to be due to the inclusion of other small similar-appearing particles in the count besides starch, probably in most cases tiny plant leucoplastids.

However, regardless of this fact the per cent starch obtained with most of these drugs when using the microscopic counting technique was found to be within the range of 1% to 2% of that obtained when using the chemical method. Thus the utility of the microscopic method is apparent.

The first drop from the pipette may then be discarded. A thorough and vigorous shaking of the suspension will aid in obtaining the desired results.

As the counting area of the Levy counting slide is 0.1 mm. in depth, starch granules will settle to different depths. Therefore, in order to make an accurate count it is necessary to use the fine adjustment of the microscope very diligently throughout each counting determination. This is of prime importance so that the starch granules in the different planes of focus can be properly counted. If this is not observed it becomes evident that inaccurate and low counts will result.

Such a microscopic counting method for the quantitative determination of starch was found to be both time- and labor-saving. This fact would be of great significance to the microscopist in the case of the above starch mixtures, if he desires to determine the amount of starch in a given mixture with a limited amount of time and effort and without resorting to the time-consuming and laborious chemical methods. However, it is felt that such a method will give most consistent results only when used for mixtures containing one type of starch and wherein most of the starch granules measure more than 10 microns in size.

SUMMARY

A study of several quantitative microscopic methods for analyzing commercial starches in mixtures and for the similar analysis of starch granules in certain vegetable drug powders has been made.

A saturated solution of zinc sulfate U. S. P. was found to be superior to agents previously used as starch-suspending agents. This suspending medium, when used with a Levy blood counting slide, gave sufficiently even starch granule dispersion to provide for more consistent microscopic starch counting than previously reported.

The above method was found best applicable to the analysis of the starches from potato tubers, grains of wheat, rice, and corn, and from the vegetable drugs belladonna, glycyrrhiza, aconite, and orris roots.

All counts and percentages ascertained were compared with results obtained from an accepted quantitative chemical analysis procedure on the same sample. Comparisons are shown in Tables II and V and were considered to be favorable.

The advantages and limitations of the procedure have been described.

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Book Reviews

Experiences with Folic Acid, by TOM D. SPIES. The Yearbook Publishers, Inc., Chicago, 1947. 110 pp. 15 x 23 cm. Price, $3.75.

That folic acid can control macrocytic anemia is now an established fact. Folic acid's place in nutrition and clinical science has changed so rapidly that a confusing literature is the result. Dr. Spies lists 80 references in this brief account.

Experiences with Folic Acid is an account of the clinical evaluation of folic acid largely by Dr. Spies and his staff. In addition to the information it contains, it could well serve as a guide to those interested in clinical research. Medical literature is so full of uncontrolled and poorly controlled clinical studies, that a scientific approach is indeed refreshing.

Since folic acid is so "new," obviously this account cannot be definitive, but it will serve as a helpful guide until research can crystallize more clearly.

Organic Analytical Reagents, by FRANK J. WELCHER. Vol. IV. D. Van Nostrand Company, New York, 1948. xiii + 624 pp. 14.5 x 23 cm. Price, $8; series price, $7.

Volume IV of *Organic Analytical Reagents* follows the same general pattern of previous volumes and shows the same meticulous care in its preparation. [For review of Volumes I and III see THIS JOURNAL, **37**, 44(1948).] The compounds covered in this volume are acidic nitro compounds, the arsonic acids, dithiocarbonates, xanthates, miscellaneous sulfur compounds, sulfonic and sulfinic acids, selenic acids, alkaloids, diazonium compounds, carbohydrates, miscellaneous natural substances, lake-forming dyestuffs, hydroxyanthraquinone and miscellaneous dyes, and dyes used in the detection of nitrites.

An index of names and synonyms of organic reagents and an index of the uses of organic reagents complete the volume.

A more helpful treatise on analytical chemistry than these four volumes has not appeared in years.

Pharmaceutical Preparations. By GEORGE E. CROSSEN and KARL J. GOLDNER. The Lea and Febiger Co., Philadelphia, 1948. 250 pp. 15.2 x 24 cm. Price $4.

The second edition of this text is a concise basic volume for students of pharmacy who have had preliminary training in chemistry and fundamental laboratory techniques and who are ready to consider official pharmaceutical preparations. It is a helpful supplementary outline of the official preparations which should be given in the course of galenical pharmacy in the colleges of pharmacy.

The authors have brought this edition into complete accord with the latest editions of the U. S. P. and the N. F. There are listed both old and new synonyms of the preparations, with added explanations for special steps which should be followed in the manufacture of some official preparations.

Each of the various classes of preparations is discussed separately in order to provide the best form of continuity. Valuable background material is furnished in the brief summaries of the Harrison Narcotic Act and the section of the Federal Food, Drug, and Cosmetic Act pertaining to "dangerous drugs" which is of interest to the pharmacist.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Assay of Heparin. II. Influence of Various Factors on Potency and Clotting Concentration of Heparin*,†,‡

By R. H. K. FOSTER

In assaying commercial samples of heparin no difference in potency was found when tested on either fresh or frozen beef or sheep plasma nor was any seasonal influence noted. Potency estimations were also unaffected by assay time or type of end point employed. The actual amount of heparin needed to inhibit coagulation is altered, however, by such factors as the state of the plasma (fresh or frozen), the aging of plasma at room temperature, the assay temperature, assay time, and type of end point. Small variations in calcium from the stoichiometrical equivalent of the citrate anticoagulant do not affect the heparin requirement. However, smaller amounts of calcium result in a lowered heparin requirement. The conditions specified for an assay must be rigidly adhered to, particularly in regard to treating the standard and unknown exactly alike.

IN THE previous paper (1) there were described the methods employed, variations seen in the character of the clot during formation and at the end point, evaluation of potency, and a comparison of the clotting curves of heparinized beef and sheep plasmas. The data presented here demonstrate the influence of environmental factors. Some general conclusions were given at the 1947 Federation meeting (2) and the data upon which these were based, together with further information, are included in this paper.

It scarcely seems necessary to state that no two batches of plasma react exactly alike. The differences are reflected in the varying amounts of heparin required for inhibition of clotting and in the character of the whole or partial clots. However, the estimated potency of heparin as determined by comparison with a standard has been found to remain unaltered. Different samples of heparin may vary in purity and potency but there is no reason for believing that commercial heparins differ in their chemical structure and we would thus expect parallel reactions in all plasmas.

The plasmas employed, beef and sheep, were prepared as previously described.[1] Some were

* Received December 2, 1947, from the Department of Pharmacology, St. Louis University School of Medicine, St. Louis 4, Mo.

† Supported by a grant from the United States Public Health Service.

‡ Acknowledgment is herewith made to Hoffman-La-Roche, Inc., Nutley, N. J., The Connaught Laboratories, Toronto, and the Wilson Laboratories, Chicago, for the specimens of heparin used.

[1] All plasmas used for obtaining the present information were "slow frozen" by placing the bottles in a General Electric deep freezer, except for the experiments illustrated in Figs. 1 and 2. Plasmas for Expts. 8 and 21 were "quick frozen" in a dry ice-acetone bath and for Expt. 9 "slow frozen." The latter were stored in a box containing dry ice.

employed in the fresh state and others were frozen and stored at about −25°. For use the frozen plasmas were thawed by immersion in the 37° water bath with frequent rotation of the bottle. They were strained through glass wool. Some specimens, especially among many samples of sheep plasma, had flocculent particles not seen in the original plasma. In most cases the flocculent particles "melted" to a transparent state and then dissolved or dispersed during the rise in temperature. Resolution of the particles nearly always occurred in beef plasma but sometimes an appreciable amount of the precipitate in a sheep plasma did not redissolve.

In assessing the influence of the several factors the distinction must be made between potency, meaning the strength of heparin as compared to a standard tested simultaneously, and the dose or concentration, meaning the actual amount of heparin required to inhibit coagulation. In Part I there are given data demonstrating the lack of effect of environmental factors on potency and in Part II the influence of these factors in causing a variation in the amount of heparin required. In some cases the same experiment provided information for both assessments.

PART I. THE STUDY OF VARIOUS FACTORS IN RELATION TO POTENCY

Comparison of Fresh and Frozen Beef and Sheep Plasmas.—Frozen plasma offers several advantages over fresh plasma. It can be prepared at any convenient time in advance of the assays and in sufficient quantity for many tests. Plasmas from several animals may be pooled so a large uniform batch is available. The behavior is established in the first experiment and this experience is available for the future tests on the batch. With fresh plasma the clotting range must be determined each time. The use of frozen plasma has previously been reported by Kuizenga, Nelson, and Cartland (3) and by Wolfrom and McNeely (4), but they made no comparison with fresh plasma. In Table I there are listed the data obtained in assays on the same lots of fresh and frozen plasmas. Blood for these tests was collected during the latter part of July and early August.

The results demonstrate no significant differences in potency when beef or sheep plasma is employed in either the fresh or the frozen state. Except in one case the P values all indicate a high degree of significance to the agreement. In the one case of divergence, the assay of heparin No. 3 on fresh and frozen sheep plasma, the low value for P, 0.02, is due not only to the difference between the means but also to the very low standard error in Expt. 67, namely, 0.2%. The same comparison made in Table II resulted in a P value of 0.48. All other values for the same sample agree statistically.

In assays using fresh sheep plasma more difficulty was encountered in determining the heparin concentration range than in using fresh beef plasma. This may have been due partly to a slower clotting reaction, for density measurements with the electrophotometer suggest this, but more likely it was largely due to the much lower density of clots from sheep plasma. In an experiment with fresh sheep plasma preceding Expt. 67, the concentration range was higher than indicated by the preliminary test and in Expt. 67 illustrated in Table I heparin No. 4 fell at the extreme edge of the range and accurate estimation of the CC_{50}[2] was not possible. This difficulty was considerably less with beef plasma or with either plasma in the frozen state.

Table II gives data obtained in earlier experiments performed on plasma from blood collected in January. These results are in fair agreement with those of Table I. In the series on fresh beef plasma, two of the results gave abnormally high standard errors; however, on each of these only two series of tubes could be used for the estimation rather than the usual four.

Tables I and II both show that even disregarding the three abnormally high standard errors obtained on beef plasma there was still a tendency for slightly better uniformity in assays made on either fresh or frozen sheep plasma. The magnitude of this better uniformity is not great, however.

The results in the two tables also indicate no difference in estimated potencies when winter and summer plasmas are employed or when the assays are carried out at a high room temperature.

Assay Time and Type of End Point.—The actual assay time evidently has no influence on potency provided the standard and unknown are read at corresponding times. This is shown in Table III where the same tubes were read at one hour and three hours. The original Reinert and Winterstein method (6) proposed four hours. In the previous paper (1) a one-hour observation time was suggested. However, when a large amount of material is being tested or if an individual is working alone one hour may be insufficient to prepare all tubes before the first ones must be read. The results of Table I are all based on a two-hour assay; those in Table II on a one-hour assay.

The two end points compared were the all-or-none and the dose-response. The all-or-none end point represents that amount of heparin which inhibits coagulation to such an extent that the plasma will just flow when the tube is tilted carefully without tapping. The dose-response end point represents the amount of heparin which allows the formation of a 50% clot. The terms used to represent these are respectively CC_{0+}, meaning a "clotting concentration" of heparin which permits the formation of slightly more than zero clot, and CC_{50}, meaning the concentration resulting in a 50% clot.[2]

With sheep plasma both end points could be read on the same tubes (Expt. 52). The scatter in the actual potencies obtained is small and the standard errors are all consistently small. The P values

[2] CC_{50} refers to "50% clotting concentration," that is, the amount of heparin that will allow the formation of a 50% clot. In the previous paper the term CD_{50}, meaning "50% clotting dose," was used. It seems more appropriate to refer to "50% clotting concentration" as in (5) since an *in vitro* system is employed. The estimation of the degree of clot formation by visual inspection is a practical means of arriving at a definite end point and it has been considered that it represents a definite proportion of the entire amount of fibrin that may be formed. The extent of conformity to this presumption is under investigation.

TABLE I.—COMPARISON OF FRESH AND FROZEN BEEF AND SHEEP PLASMAS

Plasmas prepared in summer.
Potencies expressed in Provisional International Units per milligram of heparin.
Toronto heparin (137 U/mg.) used as standard. (Potency determined by checking against the Provisional International Standard stated to contain 130 U/mg.)
Under Plasma Column, B = Beef, S = Sheep.

Heparin Sample		1			2			3			4			
Expt.	Plasma	U/Mg.	S_E, %	P	U/Mg.	S_E, %	P	U/Mg.	S_E, %	P	U/Mg.	S_E, %	P	N
64	BQ-Fresh	100.4	2.2		101.5	0.9		97.6	0.7		91.4	1.9		4
				0.51			0.38			0.44			0.16	
65	BQ-Frozen	98.5	2.8		99.5	2.7		96.0	2.0		87.2	5.0		4
67	SO-Fresh	98.5	1.1		102.9	0.4		95.1	0.2		<90.0	...	...	4
				0.19			0.85			0.02				
68	SO-Frozen	101.0	1.2		102.7	0.9		98.9	1.0		88.0	0.8	...	4
65–68	Comparison of frozen plasmas	...	...	0.30	...	...	0.19	...	...	0.21	...	...	0.74	...

Frozen plasmas were tested 24 hours after preparation.
S_E = standard error of the mean.
P = probability of agreement.
Assay temperature: 37°. Assay time: 2 hours.
Room temperatures: Expt. 64, 32°; Expt. 65, 27°; Expt. 67 and 68, 34° to 36°.

TABLE II.—COMPARISON OF FRESH AND FROZEN BEEF AND SHEEP PLASMAS

Plasmas prepared in winter.
Potencies expressed in Provisional International Units per milligram of heparin.
Toronto heparin (137 U/mg.) used as standard.

Heparin Sample		1			2			3			
Expt.	Plasma	U/Mg.	S_E, %	P	U/Mg.	S_E, %	P	U/Mg.	S_E, %	P	N
37	BJ-Fresh	105.0	10.4		104.2	1.2		99.9	5.8		2
				0.14			0.25			0.53	
40	BJ-Frozen	98.9	2.1		101.8	1.1		100.8	0.7		4
				0.16			0.14			0.05	
41	BJ-Frozen	95.1	2.4		105.3	2.2		95.1	3.6		4
38	SF-Fresh	100.2	0.9		105.1	1.1		100.3	0.3		4
				0.31			0.43			0.48	
42	SF-Frozen	98.7	0.3		103.3	1.8		99.8	0.3		3
40–42	Comparison of frozen plasmas	...	...	0.93	...	...	0.44	...	...	0.46	.
41–42	Comparison of frozen plasmas	...	...	0.12	...	...	0.48	...	...	0.12	.

Frozen beef plasma tested 7 days after preparation.
Frozen sheep plasmas tested 12 days after preparation.
Assay temperature: 37°; assay time 1 hour.
Room temperature: 22° to 25°.

were not calculated, but by inspection one would expect to find them about the same as those in Tables I and II.

With beef plasma the two end points could not be read on the same series of tubes so two separate assays were made (Expt. 49 and 50). The spread in the estimated potencies is no greater than those estimated on sheep plasma or than the potencies given in the first two tables. By both end points the standard errors are higher than with sheep plasma. The errors with the all-or-none end point are higher in two of the three examples than are those by the dose-response end point. Although this is in accord with theoretical consideration of the nature of the two end points and the manner of determining them, practically, it does not seem to matter a great deal since with either plasma or with either end point the errors are lower than the assay errors

cited by other reports in the literature. In almost any work with biological material even for bioassays a standard error of 5% is considered not excessive. The *in vitro* assay of heparin is more akin to a chemical titration than to a "bio" assay and lower standard errors should be expected, but the vagaries of the very labile plasma make one suspect it still behaves as a "living" substance.

The all-or-none end point was used by Kuizenga, *et al.* (3), for plasma and by most other workers using whole blood. Jaques and Charles (7) were able to obtain an approximate dose-response end point with blood.

At this point it should be mentioned that a minor modification in procedure has recently been introduced which helps reduce the errors slightly. When several unknowns are being tested at the same time an extra set of standard tubes is run at the beginning of the first series. The standard is also run at the end of each series. The results on the standard at the beginning and end of the first series are averaged for calculating the potencies of the intervening unknowns. The standard at the end of the first series also serves as the standard for the beginning of the second series and is averaged with the standard at the end of this series, and so on through the four series of tubes. All potencies in Tables I to III were estimated by this technique except in Expts. 37 and 38 of Table II. In one experiment that was checked the standard errors were about 0.3% lower by this method than by calculating the results on the basis of only the standards at the ends of the series. This difference is hardly consequential but because of the gradual change of plasma (see Part II) it seems preferable to employ the standard in duplicate for each series, which is made possible merely by adding one extra set of five tubes at the beginning of the assay.

PART II. INFLUENCE OF THE VARIOUS FACTORS ON THE AMOUNT OF HEPARIN REQUIRED FOR INHIBITION

Comparison of Fresh and Frozen Plasma.—Tests were conducted on fresh plasma immediately after centrifugation. A portion of the same plasma was placed in the freezer and tested the next day or several days later. Storage time in the frozen state did not seem to be a significant factor although no systematic study of this was made. Taylor, *et al.* (8), have shown that with frozen human citrated plasma the clotting elements remain substantially unchanged for six months, except prothrombin, which is still present in effective quantity (40% of original).

The major changes evidently occur before or during the initial process of freezing and the subsequent thawing. Taylor, *et al.*, also showed that slow thawing (overnight) caused a rather prominent decrease in prothrombin in human plasma.

Table IV shows the comparison between the fresh and frozen plasmas. In every case frozen plasma required more heparin than the original fresh plasma. Frozen sheep plasma required only slightly more heparin but frozen beef plasma required considerably more, the increases averaging about 10% and 70%, respectively.

The data of Table IV also illustrate both the species and individual differences. In the frozen state, sheep plasma required about 75% more heparin than beef plasma while in the fresh state about 150% more. Comparison between individual cases shows a wide spread in the sheep-beef heparin concentration ratio, e.g., 1.14, 1.49, 2.07, and 2.72 for the four possible ratios among the frozen beef and sheep plasmas using heparin No. 1 as the example; that is, in one case the beef and sheep plasmas required almost the same amount of heparin, but in another case the sheep plasma required nearly three times as much.

Effect of Aging After Thawing.—The amount of heparin required to inhibit coagulation is reduced as plasma stands at room temperature after thawing. This is illustrated by data plotted in Fig. 1. Even

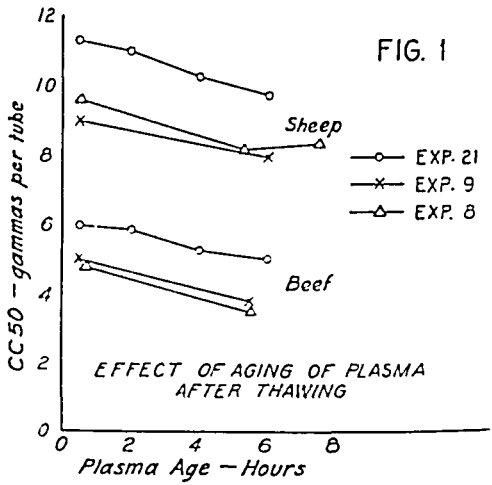

Fig. 1.—The curves show gradually lowered heparin requirements over a period of hours. Aging took place at room temperature.

in regular assays the CC_{50} may go down slightly during the relatively short time required for filling all series of tubes. Picking a few cases at random from different experiments on both plasmas the variations in dosage were:

First test:	3.4	5.2	6.0	3.9	7.9	7.7	5.4
Fourth test:	3.5	5.2	5.7	3.6	7.4	7.1	4.6

The time intervals for preparing the series of tubes from which these values were obtained ran from about three-fourths to one and one-half hours. Intervals as long as the latter did not consistently result in a decrease in the CC_{50} since technical errors or other factors can play a big role in such slight variations. Nevertheless the trend was unmistakable from the above values and from Fig. 1. They indicate the desirability of duplicating the standard heparin for each series as described in Part I, except when no more than one or two unknowns are being run.

The change in the plasma is probably explained by the loss of fibrinogen on standing. Using the electrophotometer the density of clots from unheparinized plasma was shown to be considerably decreased after a few hours' standing. Lavergne

TABLE III.—COMPARISON OF DIFFERENT END POINTS AND OBSERVATION TIMES

Frozen plasmas employed.
Potencies expressed in Provisional International Units per milligram.
Standard: see below.

Heparin Sample				1		2		3		4		
Expt.	Plasma	Obs. time, hours	End Point	U/Mg.	S_E, %	U/Mg.	S_E, %	Mg.	S_E, %	U/Mg.	S_E, %	N
49	BI	1	CC_{0+}	98.0	4.7	103.0	2.7	100.3	5.1	..	...	4
49	BI	3	CC_{0+}	95.4	6.2	102.3	1.7	102.8	4.5	..	...	4
52	SF	1	CC_{0+}	(100)[a]	...	99.8	0.7	95.5	0.8	90.5	0.8	4
52	SF	3	CC_{0+}	(100)[a]	...	101.2	0.8	97.0	1.2	90.8	0.7	4
50	BI	1	CC_{50}	(100)[a]	...	97.6	2.8	101.0	2.3	94.6	1.4	4
50	BI	3	CC_{50}	(100)[a]	...	101.7	3.0	102.0	2.5	95.0	1.1	4
52	SF	1	CC_{50}	(100)[a]	...	103.1	0.4	98.4	0.6	93.9	1.3	4
52	SF	3	CC_{50}	(100)[a]	...	102.1	0.3	97.7	0.8	92.2	0.8	4

[a] Heparin No. 1 used as standard. In Expt. 49 Toronto heparin (137 U/mg.) was used as standard.
CC_{0+} = all-or-none end point; slight amount of fibrin present and plasma just flows when tube is tilted.
CC_{50} = dose-response end point; 50% fibrin present (estimated).

TABLE IV.—INFLUENCE OF SPECIES AND STATE OF PLASMA ON CONCENTRATION OF HEPARIN REQUIRED

One-hour observation time.

Heparin Sample			1		2		3		4		5*		
			CC_{50}		CC_{50}		CC_{50}		CC_{50}		CC_{50}		Average
Expt.	Plasma	N	γ	Ratio	γ	Ratio	γ	Ratio	γ	Ratio	γ	Ratio	Ratio
65	BQ-Frozen	4	5.1		5.0		5.2		5.7		3.6		
				1.50		1.52		1.48		1.54		1.44	1.50
64	BQ-Fresh-	4	3.4		3.3		3.5		3.7		2.5		
41	BJ-Frozen	4	2.8		2.5		2.8		...		1.9		
				1.87		1.92		2.00				1.90	1.92
37	BJ-Fresh	2	1.5		1.3		1.4		...		1.0		
68	SO-Frozen	4	7.6		7.5		7.8		8.7		5.8		
				1.06		1.07		1.04		>1.11		1.11	1.07
67	SO-Fresh	4	7.2		7.0		7.5		>7.8		5.2		
42	SF-Frozen	3	5.8		5.5		5.8		...		4.1		
				1.11		1.10		1.11				1.08	1.10
38	SF-Fresh	4	5.2		5.0		5.2		...		3.8		
Average: Frozen Sheep			6.7		6.5		6.8		...		4.95		
				1.74		1.73		1.70				1.80	1.74
Average: Frozen Beef			3.85		3.75		4.0		...		2.75		
Average: Fresh Sheep			6.2		6.0		6.35		...		4.5		
				2.53		2.61		2.70				2.57	2.60
Average: Fresh Beef			2.45		2.3		2.45		...		1.75		

CC_{50} = 50% clotting concentration of heparin in gammas per tube (= 2 cc. solution ≈ 1 cc. pf plasma).
* Toronto heparin.

and Lavergne-Poindessault (9) demonstrated a loss of fibrinogen but no loss of prothrombin in human plasma allowed to stand twenty-four hours at 0°. Taylor, *et al.* (8), could demonstrate little or no fibrinogen in human plasma stored at room temperature for one to six months, although 2% prothrombin was present even after six months.

Effect of Assay Temperature, End Point, and Observation Time.—An experiment was set up to study these factors simultaneously. For the different observation times, one and three hours, the same tubes were observed. With sheep plasma it was also possible to observe both end points in the same series of tubes. With beef plasma the CC_{0+} could not be observed in the same series of tubes from which the CC_{50} was estimated because of the great difference in dosage ranges. Separate series were set up to be run at room temperature and in the water bath at 37°.

The difference in dosage ranges for the CC_{50} and CC_{0+} with beef plasma may be described as follows. The main part of the clotting curve is quite parallel to the curve for sheep plasma. However, as the heparin concentration is increased and the lower portion of the curve reached the curve begins to flatten out. This is illustrated in the figure in the

previous paper (1). With the beef plasmas here used small amounts of coagulum were seen to persist beyond all proportion to the increasing amounts of heparin added. While these persistent "traces" of coagulum have no influence on the CC_{10} they have a marked effect on the CC_{0+} when they form a pellicle at or near the surface to prevent flow on tilting. Beef plasmas apparently vary considerably in their tendency to produce these persistent traces of clot as is evident by comparing the CC_{0+} values in Table V with the published curve. This curve, for beef plasma, reaches the base line (zero clot) with the heparin concentration about 15% above the CC_{10}. In Table V the CC_{0+} figures are 150% higher than the CC_{10}'s. The difference seen between the two end points had no significant effect on the potency estimation as has been mentioned.

heparin which would produce clotting within a specified time. This technique was previously used by the author (5). In assays then, the amounts of calcium were always suboptimal so that the response of the plasma was affected by volumetric errors in measuring the calcium chloride as well as by those in measuring the heparin and plasma itself. Such errors, from these three sources, are not merely dilution errors as would be with the saline solution, but they occur at the critical range of the clotting reaction and may profoundly affect the reaction. It therefore seems more logical to recalcify with an amount of calcium stoichiometrically equivalent to the sodium citrate present. Within limits well within those beyond which volumetric errors of calcium could seriously throw off the results, supraoptimal amounts of calcium have little effect on the coagulation. This is because recalcification with

TABLE V.—INFLUENCE OF TEMPERATURE, OBSERVATION TIME, AND END POINT ON CONCENTRATION OF HEPARIN REQUIRED

Frozen plasmas employed.
Heparin No. 1 used for all tests.

Expt.	Plasma	End Point	37° Observation times 1 hour		37° Observation times 3 hours		22-25° Observation times 1 hour		22-25° Observation times 3 hours		N
			γ	γ	γ	γ	γ	γ	γ	γ	
46	BF	CC_{10}	3.8	3.8	3.9	3.8	3.9	3.9	4.2	4.1	4
46	BF	CC_{0+}	>5.1		>5.1		>5.1		>5.1		4
49	BI*	CC_{0+}	9.4		9.4		...		...		4
45	SE	CC_{10}	5.8	5.8	6.1	6.1	5.5	5.5	5.9	5.9	4
45	SE	CC_{0+}	6.3	6.3	6.5	6.5	5.9	6.0	6.5	6.4	4

* Plasma BI was substituted since the supply of BF was used up. The CC_{10} of heparin No. 1 using plasma BI was 3.7γ at 37° assay temperature and 1 hour observation time. The plasma was thus similar to plasma BF.

In the case of sheep plasmas the CC_{0+} required only a slightly higher dose of heparin than the CC_{10}. In Table V the greatest difference is 10%. The published curve for sheep plasma reaches the base line at 6% higher concentration. Thus even with sheep plasma the spread in the clotting curve is somewhat greater than previously obtained.

The effect of prolonging the observation time from one hour to three hours was to raise the required amount of heparin, using either end point. The effect was slightly greater with sheep than with beef plasma. For both species the effect of longer observation time in raising the required concentration was greater at room temperature than at 37°. These results reflect the continued growth of the clot. This has been demonstrated by the electrophotometer, most clots continuing to increase in density for twenty-four hours.

The effect of raising the assay temperature from room temperature to 37° is slight though greater on sheep than on beef plasma. Both this and the previous observation suggest that beef plasma clots somewhat faster than sheep plasma. In carrying out assays at the two temperatures the clots of both plasmas were more "readable" at the higher assay temperature.

Effect of Calcium Concentration.—The original method of Reinert and Winterstein (6) specified a test for "standardization of plasma" before assaying heparin. The purpose of the test was to determine the amount of calcium necessary for recalcification in the presence of a standard amount of

2 mg. of calcium chloride provides an excess beyond the amount needed in the clotting reaction.[3]

Tests were made using 1, 2, and 3 mg. of calcium chloride for neutralizing the citrate, the results being illustrated in Fig. 2. The plasmas used for these tests, as well as all other plasmas in the work, contained 4 mg. of sodium citrate per milliliter,[4] and the amount of calcium chloride stoichiometrically equivalent to this is approximately 2 mg. (theoretical 1.865 mg.). The amount of heparin required was twice that when only 1 mg. of calcium chloride was used, but there was no difference in heparin requirements between 2 and 3 mg. of calcium chloride.

With some plasmas 1 mg. of calcium chloride was apparently close to sufficient for recalcification since some of the clotting curves were very steep. Below 1 mg. there was obtained a rapid diminution in coagulability as the amount was reduced. Concentrations between 2 and 3 mg. were not tried but it hardly seems likely that any modification of the curves of Fig. 2 would be found.

It may be remarked that in assays conducted over

[3] Two milligrams of calcium chloride per milliliter of citrated plasma was the concentration used by Kuizenga, *et al.* (3).

[4] In collecting blood conditions generally necessitate having the slaughter-house butcher fill the bottles and he usually does not succeed in filling just to the mark. Over-filling is more likely. As much as 15% excess blood, reducing the sodium citrate concentration to 3.5 mg./ml., has caused no noticeable effect on results. The filling error is seldom over 5%, however.

several years the amount of calcium chloride varied from about 0.7 to 1.2 mg. with different fresh beef plasmas. Since changing to 2 mg. for recalcification the standard errors have been rather consistently lower.

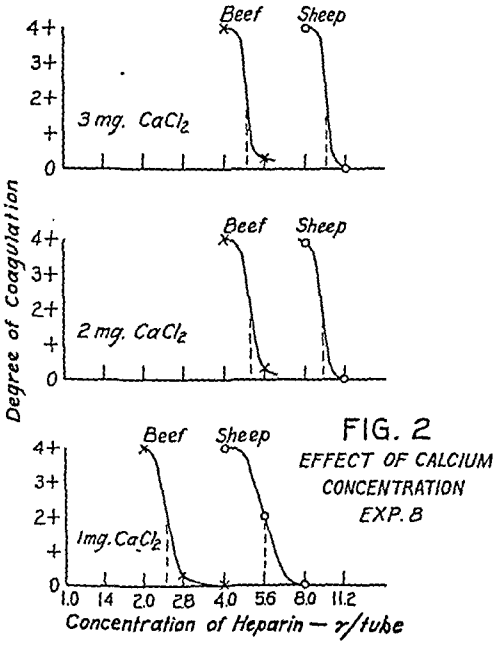

FIG. 2
EFFECT OF CALCIUM CONCENTRATION
EXP. 8

Fig. 2.—Influence of re-calcification with different amounts of calcium chloride on the CC_{50} of heparin. The 50% points (2+) are indicated by dotted lines.

DISCUSSION

The work discussed in this and the preceding papers (1, 2) was undertaken to aid the Committee on Revision of the Pharmacopœia of the United States in setting up an official assay method for heparin. An official method has not yet been decided on but will be following a collaborative study program set up by the Committee on Revision. These particular experiments were designed primarily to compare the use of beef plasma and sheep plasma and were extended to show the effects of various environmental factors.

The results of Part I demonstrate that the potencies of several samples of heparin, estimated by comparing with a standard run concurrently, do not vary with the conditions employed here. The selection of plasma rather than whole blood for the assay medium seems to be particularly that of practicality. It is apparent that for control purposes it is a great advantage to have on hand a large stock of suitable material readily available for use. Stored frozen plasma meets this need. Several liters may be mixed and frozen at one time so that a uniform batch with known clotting characteristics is available. In addition there is a considerable saving in time.

Jalling, Jorpes, and Linden (10) have discussed the use of various clotting systems, including whole blood. They point out that heparin may be partly neutralized by reacting with prothrombin, thrombin, and thromboplastin and that any method which involves this neutralization by only one of these constituents entails some risk. They object to citrated or oxalated plasma (which contains the above three elements) only on the grounds that an excess of calcium ions seriously disturbs the coagulation. This objection has been met by the results illustrated in Fig. 2, which showed that an excess of calcium of at least 50% caused no alteration in the concentration of heparin required. In conclusion they recommend fresh whole blood.

It is possible that different samples of heparin may react differently with the different constituents of the blood and the synthetic anticoagulants in comparison with heparin have been shown to have a different action on plasma in contrast to that on blood (11). Also Jaques and Charles (7) showed differences in relative activity of heparin samples assayed on whole blood and on plasma.

Although these objections may be valid they do not appear too important to the author when it comes to the problem of pharmaceutical control. Although beef heparin has been purified to different degrees there is no evidence that any commercial heparins behave differently from each other in their fundamental reactions.

The development of synthetic anticoagulants and their assay is of no immediate concern to the problem of an official assay method for heparin. Nor is it necessary that a single assay method meet the needs of all types of anticoagulants.

We may summarize the advantages of plasma (beef or sheep) as follows:

1. A large amount of uniform plasma (mixture of several batches) may be kept on hand in the frozen state. This is a practical time-saving point.
2. Once tested, the clotting range of this plasma is known and preliminary tests need not be run. Results can be more easily verified.
3. The degree of coagulation is more easily and more accurately estimated on plasma than on whole blood.
4. Plasma volumes can be measured more accurately and more quickly than blood volumes.
5. Plasma has already been proved to be a satisfactory assay medium and is practically exclusively used by all manufacturers in this country.
6. On occasion frozen plasma can be shipped while fresh blood deteriorates too rapidly.

The influence of the various factors on the actual concentration of heparin required demands less attention than their possible effect on the potency determinations on account of the use of a standard. Nevertheless, they are important in evaluating and planning the technique of the assay since it is important to know how the heparin concentration range may be shifted and, of course, those conditions must be selected which would tend to cause the least variation during the progress of the assay. Most of these factors need little discussion beyond that given in Part II with the results.

Data showing the effect of season were not tabulated as they were deemed too inconclusive to present in organized form. There was no seasonal influence on potency but there was an apparent seasonal effect on the concentration of heparin re-

quired. Since there may be marked individual variations the seasonal trend was not clearcut. There was a tendency for both beef and sheep plasmas to require the most heparin during spring and the least in midwinter. Jorpes and his co-workers (10) found the greatest requirement in summer and the least in winter. The heparin concentration range they found for whole blood was about fourfold, whereas in this work the extremes for any two individual plasmas was only slightly more than twofold for both beef and sheep. It was also noted that winter plasma, particularly beef, clots more homogeneously than that obtained during other seasons.

In the previous paper the statement was made that "a fully clotted sample is one which is as dense as the clot in a tube containing no heparin." This is not strictly correct, for by electrophotometer measurements it was noted that clots from unheparinized plasma were less dense than those containing subminimal amounts of heparin. This can be seen visually but neither the significance nor degree was appreciated prior to the measurements on optical density. The clots of greatest density were in those tubes in which full coagulation, even though delayed, finally occurred. A report on this is to be made elsewhere.

A word is necessary to emphasize that the significance of statistical measurement should not be overrated. The standard errors represent "titration" errors only in the group of solutions as used at the time. They cannot correctly be construed to signify that the true potency is within the limits implied by the standard error. The errors are of course magnified beyond the range seen in ordinary chemical titrations because of the nature of the clotting reaction. The example, in Table I, Expts 67 and 68, using fresh and frozen sheep plasma, where the potency of heparin No. 3 was estimated as 95.1 U/mg. (Provisional International Units per milligram) and 98.9 U/mg., respectively, serves to illustrate. The P value 0.02 showed these results to be significantly different. The standard errors were 0.2 per cent and 1.0 per cent, respectively. The same solution was used for both tests. In Table II, Expts. 38 and 42, the same sample of heparin but a different solution, also tested on both fresh and frozen sheep plasma, was found to contain 100.3 U/mg. and 99.7 U/mg., respectively, with standard errors of 0.3 per cent and 0.3 per cent and a P value of 0.48 indicating excellent agreement between the results. The P value of 0.02 in the former case indicates a 50 to 1 chance that the two results are significantly different. Since the earlier comparison indicated very little difference, and this in the opposite direction, we must conclude that some unknown factor or factors caused the apparent discrepancy. The comment of Wallace and Snedecor (12) on correlation should be recalled, substituting "statistical" for "correlation" to cover this as well as all other fields: "Unless hard thinking and common sense are used in interpretation, statistical work may do more harm than good."

CONCLUSIONS

1. In assaying commercial samples of heparin no difference in potency was found when tested on either fresh or frozen beef or sheep plasma, nor was any seasonal influence noted.

2. Potency estimations were also unaffected by assay time or type of end-point employed.

3. The actual amount of heparin needed to inhibit coagulation is however altered by such factors as the state of the plasma (fresh or frozen), the aging of plasma at room temperature, the assay temperature, assay time and type of end-point.

4. Small variations in calcium from the stoichiometrical equivalent of the citrate anticoagulant do not affect the heparin requirement. However smaller amounts of calcium result in a lowered heparin requirement.

5. The conditions specified for an assay must be rigidly adhered to, particularly in regard to treating the standard and unknown exactly alike.

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A Pharmacological Study of *dl*-Desoxyephedrine Hydrochloride*

By THOMAS J. HALEY†,‡

The LD_{50} (intraperitoneal) of *dl*-desoxyephedrine hydrochloride for rats and mice was determined to be 54.48 and 61.16 mg./Kg., respectively. The oral LD_{50} for mice was 143 mg./Kg. Chronic toxicity data following intraperitoneal injections in rats are reported. Effects of the drug on the isolated uteri and on the isolated ileum of both mice and guinea pigs were determined. The drug counteracted both pilocarpine and barium spasm of the intestine of these animals. Body temperature was elevated by small doses of *dl*-desoxyephedrine. The compound usually stimulated respiration and caused hypotension in anesthetized rabbits. In dogs the pressor effect was only about $^1/_{500}$ that of epinephrine.

IN A RECENT review of the literature of desoxyephedrine, Haley (1) pointed out that, although the dextro isomer had been extensively studied, little was known concerning the racemic form of the drug. Inasmuch as clinical usage of the racemate had recently commenced, it was thought that a pharmacological and toxicological study might reveal some definite information about the mode of action of the drug.

Graham, Cartland, and Woodruff (2), using the U. S. P. dog assay, found that in pressor activity *dl*-desoxyephedrine hydrochloride was only $^1/_{275}$ as potent as epinephrine. These investigators found that the drug had a dilator effect upon the isolated perfused rabbit lung preparation. Lands, Nash, Granger, and Dertinger (3) found that in dogs the pressor effect of the drug was $^1/_{200}$ that of epinephrine. Swanson, Scott, Lee, and Chen (4) found that in the decerebrated and pithed dog the *dl*-form of the drug was intermediate in pressor activity, with the *l*-isomer showing the greatest pressor effect of the three forms of the drug. However, Hauschild (5) found that the pressor effect in cats was equal when equal doses (0.02 mg./Kg.) of all forms of the drug were administered intravenously. All the above investigators reported that tachyphylaxis developed upon continued administration of the drug. Domenjoz and Fleisch (6) have shown that *dl*- and *d*-desoxyephedrine are about equal in vasoconstrictor activity in cats. Turnbull, Hamilton, Simon, and George (7) found that 0.125 per cent *dl*-desoxyephedrine hydrochloride had a prolonged vasoconstrictor action on the human nasal mucosa. Further, there was no histological evidence of tissue damage. Novelli and Tainter (8) have shown that the *dl*-form is less potent as a central motor stimulant than the *d*-isomer when measured upon rats in the jiggle cage. Graham, *et al.* (2), found that the intravenous LD_{50} of the racemate in large rats was 20 mg./Kg. Lands, *et al.* (3), using a small number of mice found that the approximate intraperitoneal LD_{50} of the *dl*-compound was 70 mg./Kg.

EXPERIMENTAL

Acute Toxicity.—A total of 110 male white rats of the Wistar strain and a total of 98 white mice of both sexes were employed in the determination of the LD_{50} values. The rats weighed between 212 and 428 Gm. (average 310 Gm.). The mice used in the determination of the intraperitoneal toxicity weighed 20 to 40 Gm. (average 27 Gm.) and those used in the determination of the oral toxicity weighed 21 to 36.5 Gm. (average 29 Gm.). The gastric intubation was accomplished with a blunt 18-gauge, $2^1/_2$-inch hypodermic needle; the dose was given in a volume of 0.25 to 0.5 cc. All the animals receiving the same dose were caged together and the room temperature was maintained at approximately 65° F.

The signs of acute toxicity observed after intraperitoneal and oral administration of *dl*-desoxyephedrine hydrochloride in both rats and mice were: piloerection, excitation, gnawing at the bottom of the cage, salivation, nasal hemorrhage, exophthalmos, and S-shaped tail, clonic convulsions, and death by respiratory paralysis. Death usually occurred within two to seven minutes after intraperitoneal injection and within 24 hours after oral administration. The respiratory paralysis seen was central in origin, because the muscle of the diaphragm contracted after direct electrical stimulation and after stimulation of the phrenic nerve. The convulsant action of the drug was shown to be neurotropic and not musculotropic because the S-shaped tail was abolished after sectioning the cord anterior to the base of the tail. Further, the convulsant effect on the hind limbs was also abolished by sectioning the cord anterior to the pelvic girdle. Table I gives the LD_{50} values determined in this study and calculated by the method of Bliss (9).

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TABLE I.—LD_{50} VALUES OF *dl*-DESOXYEPHEDRINE HYDROCHLORIDE IN ANIMALS

Animal	Number Used	Route	Slope	LD_{50}, Mg./Kg.	Standard Error
Rat, large	83	Intraperitoneal	24.84	54.48	±0.83
Mouse	40	Intraperitoneal	44.79	61.16	±0.34
Mouse	25	*Per os*	25.47	143.00	±0.60

TABLE II.—AVERAGE BODY WEIGHT OF RATS UNDER PROLONGED ADMINISTRATION OF *dl*-DESOXYEPHEDRINE HYDROCHLORIDE

Days	Control	Group 1, 0.5 Mg./Day	Group 2, 1.0 Mg./Day	Group 3, 1.5 Mg./Day	Group 4, 2.0 Mg./Day	Group 5, 3.0 Mg./Day
	(5)	(5)	(5)	(5)	(5)	(5)
0	223.8 Gm.	375.8 Gm.	246.6 Gm.	376.2 Gm.	248.8 Gm.	380.2 Gm.
	(5)	(4)	(5)	(5)	(5)	(5)
28	235.8 Gm.	351.0 Gm.	258.6 Gm.	351.8 Gm.	242.4 Gm.	351.4 Gm.
	(5)	(3)	(5)	(5)	(5)	(4)
63	248.0 Gm.	346.6 Gm.	275.0 Gm.	338.2 Gm.	237.6 Gm.	325.2 Gm.
	(5)	(3)	(5)	(5)	(4)	(3)
98	262.2 Gm.	349.0 Gm.	269.4 Gm.	307.8 Gm.	237.0 Gm.	295.7 Gm.
Total drug given	0	35.0 mg.	70.0 mg.	105.0 mg.	140.0 mg.	210.0 mg.
Weight gain or loss	+38.4 Gm.	−26.8 Gm.	+22.8 Gm.	−68.4 Gm.	−11.8 Gm.	−90.5 Gm.

Chronic Toxicity.—The effects of continued intraperitoneal administration of the drug in white rats in doses of 0.5, 1.0, 1.5, 2.0, and 3.0 mg. daily for five days of the week were determined over a period of 98 days. Six groups of five animals each were used. The control animals received 0.1 cc. of normal saline solution in place of the drug. The animals were weighed at the beginning of the experiment and each week thereafter. At the end of the period all survivors were sacrificed and their organs examined macroscopically.

The same symptoms of toxicity seen in the acute toxicity study were also observed in this study, except that convulsions were absent. Further, ejaculation was observed in all medicated groups at least three to four times a week. The signs of central stimulation were absent in Group 1 after the 28th day, but were seen in all other groups throughout the study.

Several animals died early in the study, but their deaths were not attributed to the drug, because of the small amount of the drug administered and the normal appearance of the organs at autopsy. One animal in Group 4 died on the 84th day and two animals in Group 5, one on the 56th day and one on the 93rd day. These deaths were due to the drug because postmortal examination showed the following: hemorrhagic lungs, intestines, and urinary bladder; lungs and liver spotted; and acute cardiac hypertrophy. The intestines were much distended with gas and the abdominal cavity gave off a putrid odor. This latter could not have been due to putrefaction because the bodies were still warm and rigor mortis had not set in when the examinations were made.

Autopsy of all the surviving animals on the 98th day showed that outside of the loss of body fat, rough skin, coarse hair, acute cardiac hypertrophy, and a slight enlargement of the adrenals, the animals were no different from the normal controls. The hearts of the medicated animals were approximately twice the size of the controls and most of the damage was in the right ventricle. This damage consisted of bands of fibrous tissue.

Table II shows the effect of continued administration of *dl*-desoxyephedrine hydrochloride on the body weight of the animals studied. The total weight loss was greatest with the larger doses of the drug. Further, the smaller animals had less weight loss than the larger ones. The medicated animals all had a decreased appetite, which could account for part of the weight loss observed. The great central stimulation, which caused excitation and rapid movement about the cage, could account for the balance of the weight loss. The weight gain in Group 2 could be explained on the basis of the age of the animals and on the lesser susceptibility of younger animals to this type of drug.

Uterine Action.—The effects of *dl*-desoxyephedrine hydrochloride on the isolated uteri of mice and guinea pigs were studied by the Magnus method. A bath holding 125 cc. of Locke-Ringer's solution and thermostatically regulated at 37.5 ± 0.1° was used. In five experiments with the guinea pig uterus, the strip was standardized to a definite height of contraction (approximately 75 mm.) using U. S. P. standard posterior pituitary solution.

The mouse uterus (twelve experiments) reacted as follows: Dilutions of 1:1,250,000 and 1:625,000 increased amplitude and decreased rate; 1:125,000 decreased amplitude and rate of contraction. The guinea-pig uterus (seven experiments) reacted as follows: Dilution of 1:1,250,000 increased amplitude and decreased rate and 1:625,000 and 1:125,000 decreased amplitude and increased rate of contraction. From these results it must be concluded that small doses of the drug are stimulatory and larger doses are inhibitory to the isolated uterus of both animals.

Intestinal Action.—The effects of the drug on the isolated ileum of mice, guinea pigs, and rabbits were studied by the Magnus method using Sollmann-Raedmaeker's solution in the same bath used for the uterine experiments. The action was studied alone

and after either pilocarpine hydrochloride or barium chloride spasm.

The mouse ileum (eight experiments) responded to dilutions of 1:1,250,000, 1:625,000, 1:250,000, and 1:125,000 of *dl*-desoxyephedrine with a decrease in amplitude and rate of contraction. The guinea-pig ileum (ten experiments) responded as follows: 1:1,250,000, 1:625,000, and 1:25,000 decreased amplitude and increased rate; 1:250,000 increased amplitude and rate; 1:125,000 increased amplitude and decreased rate, and 1:12,500 both amplitude and rate of contraction decreased. The rabbit ileum (ten experiments) responded as follows: 1:1,250,000 and 1:125,000 no change in amplitude or rate; 1:62,500 decreased amplitude and increased rate; and 1:25,000, 1:12,500, and 1:6250 decreased amplitude and rate of contraction. In all these experiments the rhythm of contraction was unchanged. In several cases, guinea-pig and rabbit ileum gave a typical sympathomimetic relaxation but it was not as pronounced or as prolonged as that given by epinephrine.

In fourteen experiments it was found that *dl*-desoxyephedrine hydrochloride in a concentration of 1:625,000 counteracted the spasm of mouse intestine produced by the addition of 1 cc. of 1:1000 of pilocarpine hydrochloride to the bath. The addition of a second dose of pilocarpine solution was without effect. In thirteen experiments using guinea-pig intestine the pilocarpine spasm was counteracted by *dl*-desoxyephedrine in a concentration of 1:6250. Here also a second dose of pilocarpine was without effect. In fourteen experiments using rabbit intestine the results were the same as with the guinea-pig intestine.

In fourteen experiments using mouse intestine, *dl*-desoxyephedrine in a concentration of 1:625,000 counteracted the effect of 1 cc. of 1:1000 barium chloride solution. A second dose of barium chloride was not effective. In six experiments barium chloride did not cause a spasm. In both the guinea pig (thirteen experiments) and the rabbit (ten experiments), *dl*-desoxyephedrine in a concentration of 1:6250 counteracted the effect of 1 cc. of 1:1000 barium chloride. In three experiments (rabbit intestine) 1:12,500 of *dl*-desoxyephedrine counteracted the barium spasm. In all cases the second dose of barium was ineffective after desoxyephedrine. However, in two experiments the rabbit intestine did not respond to barium chloride.

Effect on Body Temperature.—Five rabbits (2.66 to 3.33 Kg. body weight), that had been used for U. S. P. pyrogen testing, were injected intravenously with a standard dose of 1 mg. of *dl*-desoxyephedrine hydrochloride. The dose was given in a total volume of 10 cc. and body temperature was taken rectally at one-hour intervals over a period of five to six hours. At the same time measurements of pupil diameter were taken. In four experiments, using the same group of five animals, the temperature rise in all animals was from 0.2 to 3.6° F. (average 1.8° F.). This hyperthermia persisted over a period of four hours whereas no sign of central stimulation or mydriasis was observed.

Effect on Blood Pressure and Respiration.—Six rabbits, weighing 4 to 5 Kg., were anesthetized with sodium pentobarbital (20 mg./Kg.) and urethane (1.5 cc./Kg. of 20 per cent) given intravenously and intraperitoneally, respectively. Blood pressure was recorded from the carotid artery with a mercury manometer, respiration with a valve system connected to the trachea, and all injections were given directly into the ear vein. All injections of the drug are expressed as the total amount administered and not as mg./Kg. However, when calculated on a microgram/kilogram basis they are in the range of dosage used by Hauschild (5). In two experiments the animals were not atropinized in order to observe any cardiac side effects which might be due to the vagus nerve. These animals died of respiratory paralysis followed by cardiac standstill 25 minutes after the beginning of the experiment. In both cases a dose of 10 mg. of the drug caused a hypotension which contributed to the fatal outcome. In four atropinized animals (1 to 2 mg. of atropine sulfate), the first dose of 1 mg. of *dl*-desoxyephedrine increased blood pressure 10 to 20 mm. while the second dose of 1 mg. caused a prompt fall of 5 to 15 mm. followed by a rise of 10 to 20 mm. Doses of 5 and 10 mg. usually produced decreases of 15 to 35 mm., sometimes followed by increases up to 30 mm. Tachyphylaxis was observed in that the response to a given dose was not constant. Further, a reversal of the pressor response often occurred after the first dose of the drug. In one animal a dose of 20 mg. of Dibenamine (N,N-dibenzyl-beta-chloroethylamine), a new sympathoadrenal blocking agent, was administered prior to a dose of *dl*-desoxyephedrine. Inasmuch as there was no reversal of the desoxyephedrine pressor response after Dibenamine and because the animals used regularly gave a pressor reversal after *dl*-desoxyephedrine itself further use of Dibenamine was abandoned. It is possible that if cats or dogs had been used the Dibenamine would have given results similar to those reported by Nickerson and Goodman (10) for epinephrine. In general respiration was decreased in volume and increased in rate following desoxyephedrine administration regardless of the dose given.

A comparison of the pressor response of epinephrine and *dl*-desoxyephedrine was made in two dogs under sodium phenobarbital anesthesia (175 mg./Kg. intraperitoneally). It was found that *dl*-desoxyephedrine was about $^1/_{600}$ as potent as epinephrine. Tachyphylaxis occurred with both animals and hypotension was observed in one animal after a dose of 10 mg.

SUMMARY

1. The LD_{50} values of *dl*-desoxyephedrine hydrochloride by intraperitoneal injection in rats and mice and by oral administration in mice were determined.

2. The signs of both acute and chronic toxicity due to the drug are described and it was shown that the convulsant action is neurotropic and not musculotropic.

3. The most pronounced effect of chronic intoxication in rats was acute cardiac hypertrophy with the greatest damage being found in the right ventricle. Body weight was also affected, the larger animals showing a greater weight loss than the smaller ones. Further, the weight loss was greater with the larger doses.

4. The isolated uteri of mice and guinea pigs were stimulated by low concentrations and inhibited by higher concentrations of the drug.

5. The effects of the drug on the isolated ileum of mice, guinea pigs, and rabbits are described. Also it was shown that the drug counteracts the intestinal spasm caused by both pilocarpine hydrochloride and barium chloride and that those agents were ineffective after the administration of *dl*-desoxyephedrine.

6. *dl*-Desoxyephedrine hydrochloride has a definite hyperthermic action which is independent of its other actions.

7. It has been shown that *dl*-desoxyephedrine hydrochloride has a slight pressor effect in rabbits but in general the hypotensive effects are more pronounced. Respiration was usually decreased in amplitude and increased in rate. When compared with ephinephrine in dogs, *dl*-desoxyephedrine hydrochloride has a pressor value of approximately $^1/_{20}$.

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Reactions With Pentachloroethane. I. Hydrochlorination of Ephedrine*,†

By C. E. MILLER‡

Pentachloroethane has been the subject o investigation to find some application of this chemical to pharmacy. The activity of pentachloroethane toward amines suggested the possibility of using it as a hydrochlorinating agent for alkaloids and related products. It has been demonstrated that pentachloroethane and ephedrine employing 1,4-dioxane as a solvent react to give ephedrine hydrochloride in yields of 80 per cent.

PENTACHLOROETHANE, because of its availability and relative inertness, has enjoyed wide usage as a highly chlorinated solvent and a diluent in certain chemical reactions. From a structural consideration the compound is unique in having only one atom of hydrogen; the other valence bonds of carbon being satisfied by the chlorine atoms present in the molecule. One of the projects at this institution has been the development of some application of this versatile chemical to pharmacy. It is the purpose of this paper to present information obtained relative to the splitting out of hydrogen chloride from pentachloroethane when it is reacted with aromatic hydroxy secondary amines, of which ephedrine may be cited as a specific example. A typical run is described in detail.

EXPERIMENTAL

Sixteen and one-half grams of ephedrine (0.1 mole) and 100 cc. of redistilled 1,4-dioxane were placed in a 500-cc. three-necked flask fitted with a dropping funnel, sealed stirrer, and a reflux condenser. Gentle warming was necessary to effect solution of the ephedrine in 1,4-dioxane. To this solution was added slowly, with stirring, 20.2 Gm. (0.1 mole) of pentachloroethane. After a few minutes the clear solution became turbid, a temperature rise of about 15° was noted and the amount of precipitate increased markedly. After this initial

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temperature rise the flask and contents were heated to the boiling point of 1,4-dioxane for half an hour and then cooled to room temperature. The solid material was collected on a Büchner funnel, washed with 100 cc. of 1,4-dioxane and dried under vacuum. The resulting white needle-shaped crystals thus obtained weighed 15.5 Gm., representing a yield of 70% and, m. p. 218.9°, corrected.

Anal.—Calcd. for $C_{10}H_{16}NOCl$: N, 6.94; Cl, 17.68. Found: N, 6.89; Cl, 17.44.

The reaction proceeds without solvent, but at a somewhat slower rate. In one run using 10% excess of pentachloroethane and no solvent, the maximum precipitation had not occurred after two hours. As further proof of the reaction, there was isolated the expected unsaturated by-product, perchloroethylene, which was identified by boiling point and decolorization of bromine in chloroform.

DISCUSSION

A survey of the literature discloses little information concerning the removal of HCl from polychloroaliphatic compounds containing one atom of hydrogen adjacent to a polyhalogenated carbon atom. Ruggeberg (1) has shown that primary amines split HCl from pentachloroethane. Preliminary experimentation indicates that the more complicated aromatic nitrogenous bodies containing several benzene nuclei react very slowly with pentachloroethane in the absence of catalysts. A future paper will discuss the effect of catalysts on the hydrochlorinating action of pentachloroethane.

CONCLUSIONS

1. It has been demonstrated that ephedrine will remove HCl from pentachloroethane with and without the use of solvent.
2. The potential use of pentachloroethane as a hydrochlorinating agent has been demonstrated.
3. The expected by-product, perchloroethylene, was isolated and identified.

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The Determination of Ephedrine in Various Medicinal Combinations*,†

By W. W. HILTY and D. T. WILSON

Alternate methods for the determination of ephedrine in U. S. P. XIII and N. F. VIII preparations are suggested. Procedures for the separation and determination of ephedrine when found in combination with other medicinals such as barbiturates, acetylsalicylic acid, phenacetin, and aminophylline are described in detail.

THE PRESENT accepted methods for the assay of medicinal preparations in which ephedrine is the active ingredient or is present in combination with other medicinal agents follow two general procedures.

In the first method ephedrine is treated as a volatile alkaloid. The ephedrine base is liberated from its salt by adding an excess of sodium hydroxide and extracting with successive portions of ether. It is finally removed from the ether by extracting it with a standard acid solution. This procedure is now employed as the official U. S. P. XIII and N. F. VI I (3, 4) assay for the salts of ephedrine, tablets, ampules, syrups, solutions, and jellies containing ephedrine.

The second procedure, originally proposed by Hilty (1) and further discussed by Welsh (5), involves preliminary acid digestion and subsequent distillation of the ephedrine and any other basic nitrogenous degredation products from an alkaline medium into an excess of standard acid. This procedure is now employed as the official method of the N. F. VIII for the assay of filled capsules of ephedrine salts and filled capsules of ephedrine sulfate and phenobarbital.

We, in our laboratory, have found it necessary

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to develop methods of analysis for a large number of preparations in which ephedrine is the active ingredient in a wide variety of diluents. We have also been called upon to develop methods of analysis for preparations in which ephedrine is present in combination with other commonly associated medicinals such as barbiturates, acetylsalicylic acid, phenacetin, and aminophylline.

Holt (2) has suggested a method of separation for some of these ingredients based on the difference of their solubilities in various solvents. In our investigations we found that ephedrine salts were not sufficiently insoluble in the common solvents suggested. This resulted in values lower than the theoretical ephedrine content.

In the process of developing these methods, various separations were investigated. However, it seemed quite logical that the U. S. P. XIII general procedures for ephedrine salts might be utilized as a starting point in the separation of some of these mixtures.

The method described by Hilty is applicable to many preparations containing ephedrine as the single active ingredient and is particularly desirable when the total amount of working time is considered.

The following methods described for the salts of ephedrine, tablets, ampuls, solutions, syrups, and jellies of ephedrine and ephedrine spray are suggested as alternates for the methods described in the U. S. P. XIII and N. F. VIII for these various preparations.

In the preparation of this paper it has been considered advisable to describe separately the analysis of each combination in its complete form in order to avoid the possibility of confusion in making cross references.

EXPERIMENTAL

Procedure for Ephedrine Sulfate and Ephedrine Hydrochloride.—Transfer about 0.30 Gm. of ephedrine sulfate or ephedrine hydrochloride, previously dried for two hours at 100° and accurately weighed, to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.003302 Gm. of anhydrous ephedrine.

TABLE I—RECOVERY OF EPHEDRINE SALTS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine Sulfate, Gm.	Ephedrine Sulfate Recovered, Gm.	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.
A	0.3000	0.3000	....	....
B	0.3000	0.2996	....	....
C	....	....	0.3000	0.2997
D	....	....	0.3000	0.2994

Procedure for Tablets Ephedrine Sulfate and Ephedrine Hydrochloride.—Weigh not less than 20 tablets, reduce them to a fine powder without appreciable loss, and transfer an accurately weighed portion, equivalent to about 0.30 Gm. ephedrine sulfate or ephedrine hydrochloride, to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004034 Gm. ephedrine hydrochloride or 0.004285 Gm. ephedrine sulfate.

TABLE II.—RECOVERY OF EPHEDRINE SALTS FROM TABLETS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine Sulfate, Gm.	Ephedrine Sulfate Recovered, Gm.	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.
A	0.3240	0.3227	....	....
B	0.3240	0.3216	....	....
C	....	....	0.3240	0.3214
D	....	....	0.3240	0.3235

Procedure for Ampuls and Solutions of Ephedrine Salts.—Transfer an accurately measured portion of the ampul solution, equivalent to about 0.30 Gm. of ephedrine sulfate to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and

a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004285 Gm. ephedrine sulfate or 0.004034 Gm. ephedrine hydrochloride.

TABLE III.—RECOVERY OF EPHEDRINE SALTS FROM AMPULS AND SOLUTIONS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine Sulfate, Gm.	Ephedrine Sulfate Recovered, Gm.	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.
A	0.3000	0.2996	....	....
B	0.3000	0.2995	....	....
C	....	....	0.3000	0.2990
D	....	....	0.3000	0.3020
E	0.2500	0.2493	....	....
F	0.2500	0.2500	....	....

Procedure for Ephedrine Jellies.—Transfer an accurately weighed portion of ephedrine jelly, equivalent to about 0.25 Gm. of ephedrine, to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.003302 Gm. of anhydrous ephedrine.

TABLE IV.—RECOVERY OF EPHEDRINE FROM JELLIES WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine Gm.	Ephedrine Recovered, Gm.
A	0.2500	0.2522
B	0.2500	0.2527

Procedure for Ephedrine Spray.—Transfer an accurately measured volume of the spray, equivalent to about 0.25 Gm. ephedrine, to a 500-cc. Kjeldahl flask. Add 25 cc. of concentrated hydrochloric acid, and an antibump tube. Heat to boiling and boil under a water-cooled reflux condenser for two hours. Cool and wash down the condenser with 50 cc. distilled water followed by 50 cc. of ether and 50 cc. of petroleum benzin. Mix thoroughly and transfer the contents of the flask to a 500-cc. separatory funnel with the aid of two 25-cc. portions of ether and two 25-cc. portions of distilled water. Wash the aqueous portion with the ether-petroleum benzin mixture to remove the oily constituents and transfer the aqueous portion to a 500-cc. Kjeldahl flask. Wash the ether solution of the oily substances with two 25-cc. portions of distilled water, adding the washings to the aqueous portion in the 500-cc. Kjeldahl flask. Warm the aqueous portion in the flask on a steam bath to remove the last traces of ether. Cool, and add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T.S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.003302 Gm. of anhydrous ephedrine.

TABLE V.—RECOVERY OF EPHEDRINE FROM SPRAYS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine, Gm.	Ephedrine Recovered, Gm.
A	0.2500	0.2490
B	0.2500	0.2540

Procedure for Syrup Ephedrine Sulfate When No Other Nitrogenous Substances Are Present.—Transfer an accurately measured volume of syrup ephedrine sulfate, equivalent to about 0.30 Gm. ephedrine sulfate, to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004285 Gm. ephedrine sulfate.

TABLE VI.—RECOVERY OF EPHEDRINE SULFATE FROM SYRUPS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine, Sulphate Gm.	Ephedrine Sulphate Recovered, Gm.
A	0.1296	0.1292
B	0.1296	0.1310

Procedure for Combination of Ephedrine Salts and a Barbiturate.—Weigh at least 20 tablets and reduce them to a fine powder. Weigh accurately a portion of the powder equivalent to about 0.1 Gm. ephedrine sulfate and transfer completely to a separator and add 10 cc. of 2% sodium hydroxide. Extract with at least six 25-cc. portions of ether, collecting these extracts in a second separator. Wash the combined extracts with three 15-cc. portions of distilled water, or until these washings are no longer alkaline to litmus, collecting these washings in a third separator. Extract this wash water with 25 cc. of ether and add the ether to the combined ether extracts in the second separator. Now to this separator add 15 cc. of 0.05 *N* sulfuric acid and shake thoroughly. After the separation is complete draw the aqueous layer into a beaker. Wash the ether with two portions of distilled water, collecting these washings in the beaker, then warm on a steam bath to remove all traces of ether. Titrate the excess acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004285 Gm. ephedrine sulfate.

Combine the water wash in the third separator with the alkaline solution in the first separator. Acidify with hydrochloric acid and extract with five 25-cc. portions of a solvent consisting of one part alcohol and four parts chloroform, collecting these extracts in a second separator. Wash the combined extracts with 25 cc. diluted hydrochloric acid. Filter the chloroformic solution into a tared beaker and evaporate on a steam bath with the aid of a current of air, dry the residue at 100° for fifteen minutes, then cool and weigh as the barbituric acid.

TABLE VII.—RECOVERY OF EPHEDRINE SALTS AND BARBITURATES WHEN ASSAYED BY PROPOSED METHOD

Sample	Ephedrine Salt Gm.	Ephedrine Salt Recovered Gm.	Barbiturate Gm.	Barbiturate Recovered Gm.
A	0.0972	0.0984	0.1944	0.1944
B	0.0972	0.0963	0.1944	0.1948
C	0.0972	0.0980	0.1944	0.1952
D	0.0972	0.0968	0.1944	0.1932

Procedure for Filled Capsule Combinations of Ephedrine Hydrochloride, Aminophylline, and 'Amytal' (Iso-amyl Ethyl Barbituric Acid, Lilly).—Weigh not less than 20 capsules and determine the average weight of each. Transfer the contents of a sufficient number of average weight capsules, equivalent to approximately four grains of aminophylline, to a separator. Add 25 cc. of 2% sodium hydroxide and extract with six portions of ether, collecting these extracts in a second separator. Wash the ether extracts with two 10-cc. portions of distilled water and transfer the water to another separator. Extract the wash water with 15 cc. of ether and add the ether to the combined ether extracts and the wash water to the alkaline solution contained in the first separator. Extract the combined ether solutions with 10 cc. of 0.05 *N* sulfuric acid, accurately measured, then with successive portions of 10 cc. and 5 cc. of distilled water. Combine the sulfuric acid and water extracts in a beaker, and warm on a steam bath until the odor of ether is no longer perceptible. Cool the solution and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004034 Gm. ephedrine hydrochloride.

Render the combined alkaline solutions in the first separator acid to litmus by the addition of dilute sulfuric acid and transfer the solution to a 250-cc. beaker with the aid of small portions of distilled water. Make the solution alkaline to litmus paper by the addition of ammonium hydroxide and then add 6 cc. in excess. Add 20 cc. of 0.10 *N* silver nitrate and allow the mixture to stand until the precipitate settles. Filter the mixture through a wetted filter paper and wash the precipitate with several small portions of distilled water until the subsequent filtrate yields no test for silver nitrate. Transfer the filter paper and the precipitate to the original beaker and dissolve the precipitate in 10 cc. of concentrated nitric acid. Add 50 cc. of distilled water, cool, and add 2 cc. of ferric ammonium sulfate T. S., and titrate the residual silver with 0.10 *N* ammonium thiocyanate. Each cc. of 0.10 *N* ammonium thiocyanate is equivalent to 0.0230 Gm. aminophylline.

Transfer the filtrate obtained above to a suitable separator and render it distinctly acid to litmus by the addition of concentrated hydrochloric acid and extract with five portions of a mixture, composed of one part alcohol and four parts chloroform, collecting these extracts in a second separator. Wash the alcohol-chloroform extracts with 25 cc. of 2% hydrochloric acid and filter into a tared beaker and evaporate on a steam bath with the aid of a current of air. Dry the residue at 100° for ten minutes and weigh as 'Amytal'.

Procedure for Filled Capsule Combinations of Ephedrine Hydrochloride, Acetylsalicylic Acid, 'Amytal', and Phenacetin.—Weigh not less than 20 capsules and determine the average weight of each. Transfer the contents of sufficient average weight capsules to yield approximately seven grains of phenacetin to a separator. Add 25 cc. of 2% sodium hydroxide and extract with six portions of ether, collecting these extracts in a second separator. Wash the ether extracts with two 10-cc. portions of distilled water and transfer the water to another separator. Extract the wash water with 15 cc. of ether and add the ether to the combined ether extracts and the wash water to the alkaline solution contained in the first separator. Retain this alkaline solution for the determination of acetylsalicylic acid and 'Amytal' as described later in this method.

Extract the combined ether solutions in the second separator with 10 cc. of 0.05 *N* sulfuric acid, accurately measured, then with successive 10-cc. and 5-cc. portions of distilled water. Combine the acid and water extracts in another separator and wash with a 15-cc. portion of ether. Wash this ether extract with 10 cc. of distilled water, combining the water portion with the acid extracts collected in a beaker and the ether portions with the ether extracts retained in the separator. Warm the acid extracts in the beaker on a steam bath until the odor of ether is no longer perceptible. Cool and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004034 Gm. of ephedrine hydrochloride.

TABLE VIII.—RECOVERY OF EPHEDRINE HYDROCHLORIDE, AMINOPHYLLINE, AND 'AMYTAL' WHEN ASSAYED BY PROPOSED METHOD

Sample	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.	Aminophylline, Gm.	Aminophylline, Recovered Gm.	'Amytal', Gm.	'Amytal' Recovered, Gm.
A	0.0486	0.0486	0.2592	0.2598	0.0486	0.0489
B	0.0486	0.0485	0.2592	0.2552	0.0486	0.0488
C	0.0486	0.0483	0.2592	0.2542	0.0486	0.0489

TABLE IX.—RECOVERY OF EPHEDRINE HYDROCHLORIDE, ACETYLSALICYLIC ACID, 'AMYTAL', AND PHENACETIN WHEN ASSAYED BY PROPOSED METHOD

Sample	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.	Acetylsalicylic Acid, Gm.	Acetylsalicylic Acid Recovered, Gm.	'Amytal', Gm.	'Amytal' Recovered, Gm.	Phenacetin, Gm.	Phenacetin Recovered, Gm.
A	0.0432	0.0420	0.2592	0.2504	0.0972	0.0949	0.4536	0.4335
B	0.0432	0.0416	0.2592	0.2476	0.0972	0.0956	0.4536	0.4454
C	0.0432	0.0416	0.2592	0.2660	0.0972	0.1004	0.4536	0.4492

Transfer the combined ether extracts to a tared beaker and evaporate to dryness on a steam bath. Dry in a desiccator to constant weight and weigh as phenacetin.

Render the combined alkaline solutions in the first separator acid to litmus paper by the addition of hydrochloric acid and extract with five portions of a solvent consisting of one part alcohol and four parts chloroform. Collect these alcohol-chloroform extracts in another separator and wash with 15 cc. of 2% hydrochloric acid and discard the acid portions. Extract the chloroform solution with two 50-cc. portions of 4% sodium bicarbonate solution and then wash with 20 cc. of distilled water. Combine the sodium bicarbonate solution and the wash water and extract with two 25-cc. portions of chloroform, collecting these chloroformic extracts in the separator with the retained chloroform. Filter the chloroform extracts into a tared beaker and evaporate on a steam bath with the aid of a current of air. Dry the residue at 100° for ten minutes, cool, and weigh as 'Amytal'.

Transfer the sodium bicarbonate solution to an Erlenmeyer flask and boil for five minutes. Add 5 cc. of 20% sodium hydroxide and boil for fifteen minutes. Cool and dilute to 500 cc. with distilled water. Transfer an aliquot equivalent to approximately one grain of acetylsalicylic acid to a glass-stoppered flask. Add 25 cc. of 0.10 *N* bromine and 6 cc. hydrochloric acid and set the flask aside for thirty minutes, shaking occasionally. Add 15 cc. potassium iodide T. S. and titrate the liberated iodine with 0.10 *N* sodium thiosulfate.

Each cc. of 0.10 *N* bromine is equivalent to 0.003 Gm. acetylsalicylic acid.

The acetylsalicylic acid in the sodium bicarbonate solution should be determined as soon as possible to prevent hydrolysis.

CONCLUSIONS

Alternate assay procedures are suggested for some of the U. S. P. XIII and N. F. VIII preparations containing ephedrine alkaloid or an ephedrine salt.

Systematized procedures for the separation and analysis of medicinal preparations containing ephedrine as a common ingredient are described.

Each method is presented in full detail to avoid any possible confusion which may result from the necessity of referring from one method to another for details that might otherwise be omitted.

The results obtained by the authors in making these separations are listed in tables accompanying this article and have been found to be quite satisfactory.

In our opinion these methods provide adequate assay procedures for medicinal preparations of this type.

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A Preliminary Investigation of the Antioxidant Effect of Nordihydroguaiaretic Acid in Cod-Liver Oil*

By O. GISVOLD, F. BOPE, and C. H. ROGERS

Five-hundredths per cent nordihydroguaiaretic acid, together with 0.01 per cent ascorbyl palmitate, offered much greater protection against peroxide accumulation and destruction of vitamin A than that obtained by using 0.05 per cent nordihydroguaiaretic acid alone or smaller concentrations of the antioxidant with and without 0.01 per cent ascorbyl palmitate.

NORDIHYDROGUAIARETIC acid, isolated from *Larrea divaricata* (1), has been shown to be a powerful antioxidant for animal fats (2), particularly those from swine and beef. Furthermore, the antioxidant effect is markedly enhanced by the presence of certain synergists, such as ascorbityl palmitate, citric acid, etc. It is not so marked in vegetable oils, although considerable protection is evidenced. In general, this is true with most antioxidants.

Fish liver oils are susceptible to oxidative rancidity and are accompanied by a considerable loss of vitamin A. This type of rancidity can be avoided in a large measure without the aid of antioxidants provided proper storage conditions are employed. However, in the case of cod-liver oil, opening of the original container and subsequent exposure to air is accompanied by an immediate oxidative reaction. Therefore, consumption of the oil within a reasonable length of time is imperative from both the standpoint of preventing the formation of an undesirable rancid oil and also the destruction of its vitamin A content.

EXPERIMENTAL

Studies involving the use of nordihydroguaiaretic acid as an antioxidant in cod-liver oil were initiated because of its marked antioxidant properties in connection with animal fats. These studies were conducted upon samples of cod-liver oil stored both at room and refrigerator temperatures. This was done because accelerated tests, i.e., involving higher temperatures with or without aeration, could not be interpreted in terms of days, weeks, months, etc. Nordihydroguaiaretic acid alone, in concentrations of 0.01, 0.025, and 0.05%, and together with 0.01% ascorbyl palmitate were used. Controls were, of course, established. Ascorbyl palmitate was chosen as a type synergist because, in the concentrations used, it is readily soluble in cod-liver oil. Ten cubic centimeter samples were withdrawn once weekly to determine their peroxide contents according to the usual procedure (3). For want of an easily accessible and suitable photoelectric colorimeter, peroxide values in these preliminary studies were obtained only at definite intervals. However, the vitamin A content was determined at the end of approximately 26 weeks by the photoelectric colorimetric method using the conventional quantitative adaption of the Carr-Price antimony trichloride in chloroform color reaction with modifications described by Benham (4).

Nordihydroguaiaretic acid and ascorbyl palmitate were readily incorporated into the cod-liver oil by warming them with a small portion of the oil, cooling the same after solution had been effected and then diluting with fresh oil to the desired volume which, for these experiments, was 300 cc.

The results of the experimental work can be best expressed by graphs (Figs. 1 and 2).

DISCUSSION

The cod-liver oil used in these experiments was Parke, Davis & Co.'s high potency cod-liver oil that had an initial peroxide value of zero, and contained 2900 I. U. of vitamin A. The vitamin A content was determined colorimetrically as described above. Figure 1 represents samples stored at room temperature and the peroxide value plotted against weeks. The peroxide value is a measure of the amount of peroxide, due to oxidative rancidity, that has accumulated in the oil. It was calculated as follows (5).

$$\text{Peroxide number} = \frac{0.5\ (\text{ml. thiosulfate used in titration}) \times (\text{normality of thiosulfate}) \times 1000}{\text{wt. of fat}}$$

It will be noted that in the case of the 0.025 and 0.05 per cent, respectively, of nordihydroguaiaretic acid alone, the most significant and greatest protection is afforded up to the 20th and 18th weeks, respectively, after which time those that also contained 0.01% ascorbyl palmitate were much better protected. A sharp break in the peroxide value curves for the 0.025 and 0.05 % nordihydroguaiaretic acid occurred after the 20th week. Nordihydroguaiaretic acid 0.01 %, plus ascorbyl palmitate, 0.01 %, gave the least protection and, strange as it seems, was less stable for a period of time than the controls. On the other hand, 0.01 % nordihydroguaiaretic acid alone was slightly better than the control.

Facilities did not permit the determination of the vitamin A content of all the samples in a short

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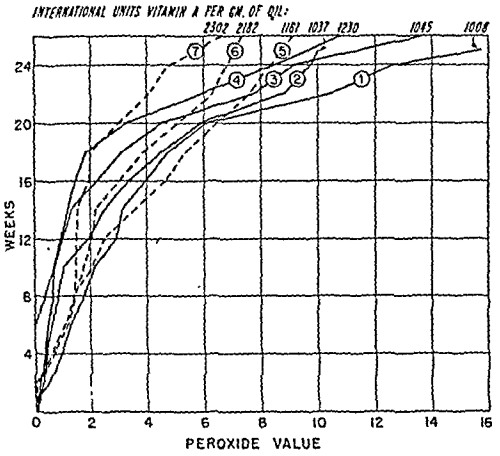

Fig. 1.—Preservative effect of varying concentrations of N. D. G. A. and of N. D. G. A. and ascorbyl palmitate on cod-liver oil stored at room temperature.

Legend: (*1*) Control (No N. D. G. A.); (*2*) 0.01% N. D. G. A.; (*3*) 0.025% N. D. G. A.; (*4*) 0.05% N. D. G. A.; (*5*) 0.01% N. D. G. A. + 0.01% ascorbyl palmitate; (*6*) 0.025% N. D. G. A. + 0.01% ascorbyl palmitate; (*7*) 0.05% N. D. G. A. + 0.01% ascorbyl palmitate.

period of time. Therefore, some samples were assayed for their vitamin A content 2 weeks later than others. If these samples had been assayed within several days, a closer correlation of the vitamin A content and time of storage would no doubt have been obtained. Nevertheless an over-all picture was gained in these preliminary studies which enabled conclusions to be drawn as to the efficacy of the antioxidant with and without the ascorbyl palmitate under the conditions of the experiment.

At about the 26th week, the samples containing 0.05 per cent nordihydroguaiaretic acid plus 0.01 per cent ascorbyl palmitate had a peroxide value of 7 and contained 2302 I. U. of vitamin A per Gm. of oil. Twenty-five thousandths per cent nordihydroguaiaretic acid, plus 0.01 per cent ascorbyl palmitate gave almost as good protection, i.e., peroxide value of 8.5 and contained 2182 I. U. of vitamin A per Gm. of oil. The control had the highest peroxide value, i.e., 15.5 and had the lowest vitamin A content, i.e., 1008 I. U. per Gm. of oil.

Figure 2 shows an almost immediate rise in peroxide value in all samples tested and after the 27th week all samples showed a much greater increase in their peroxide contents when compared to those samples stored at room temperature. However, the over-all relationships between the concentration of antioxidants to the controls when compared to the protective value of those stored at room temperature were somewhat similar. The greatest protection was evidenced in the sample containing 0.05 per cent nordihydroguaiaretic acid, plus 0.01% ascorbyl palmitate which, after the 27th week, had a peroxide value of 12.5 and contained 2445 I. U. of vitamin A per Gm. of oil. Nordihydroguaiaretic acid, 0.025 %, plus 0.01 per cent ascorbyl palmitate gave almost as good protection from the standpoint of the vitamin A content, i.e., 2230 I. U. per Gm. after the 27th week. However, the peroxide value rose to 16.5. The control had the highest peroxide content even after the 25th week, i.e., 29.5 and the lowest vitamin A content, i.e., 708 I. U. per Gm. of oil.

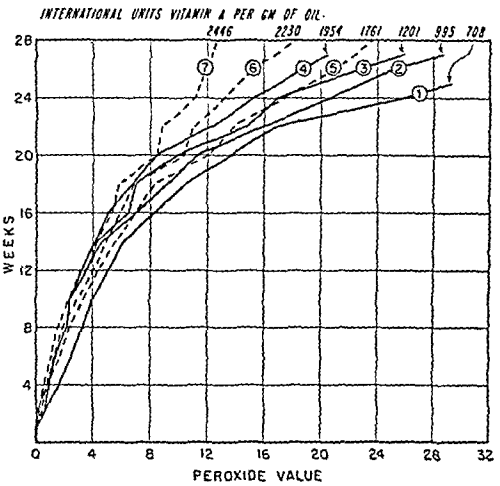

Fig. 2.—Preservative effect of varying concentrations of N. D. G. A. and of N. D. G. A. and ascorbyl palmitate on cod-liver oil stored at refrigerator temperature.

Legend: (*1*) Control (No N, D. G. A.); (*2*) 0.01% N. D. G. A.; (*3*) 0.025% N. D. G. A.; (*4*) 0.05% N. D. G. A.; (*5*) 0.01% N. D. G. A. + 0.01% ascorbyl palmitate; (*6*) 0.025% N. D. G. A. + 0.01% ascorbyl palmitate; (*7*) 0.05% N. D. G. A. + 0.01% ascorbyl palmitate.

CONCLUSIONS

(1) The peroxide value alone is not a measure of the amount of destruction of vitamin A, but must also be correlated with the temperature at which the peroxides were allowed to accumulate.

(2) The destruction of vitamin A in samples of cod-liver oil stored for 26 weeks at room temperature or in the refrigerator was essentially the same when 10-cc. portions were removed weekly and tested for their peroxide contents.

(3) Sample of cod-liver oil with or without nordihydroguaiaretic acid alone or with ascorbyl palmitate showed a greater peroxide concentration when stored in the refrigerator as compared to those stored at room temperature without a commensurate decrease in the vitamin A content after a period of about 26 to 27 weeks.

(4) Nordihydroguaiaretic acid, 0.05 per cent, together with 0.01 per cent ascorbyl palmitate,

offered much greater protection against peroxide accumulation and destruction of vitamin A than that obtained by using 0.05 per cent nordihydroguaiaretic acid alone and smaller concentrations of the antioxidant with and without ascorbyl palmitate.

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Colorimetric Determination of Peroxides in Mineral Oils*

By MILTON J. GOLDEN

A simple and accurate quantitative method is proposed by which the amount of peroxides formed in mineral oil is determined quantitatively by measuring in a Klett-Summerson photoelectric colorimeter the developing pink or red color of the oxidation of the ferrous ion to the ferric ion by the peroxides. Several mineral oil samples have been tested accordingly, and a table is given which compares the quantitative Peroxide Values of the oils with their respective Peroxide Tests. A graph is also given to facilitate the determination of the amount of peroxides in any 5 cc. of heated mineral oil. This quantitative method may be used for determining accurately the peroxide content of other oils, such as cod-liver oil or vegetable oil, and also for evaluating the effectiveness of antioxidants.

THE PRINCIPAL methods that are used today for estimating the peroxide content of such materials as cracked gasoline, hydrocarbons, oils, and fats are based upon the oxidation of either potassium iodide or a ferrous salt by the peroxides.

In 1929, Marks and Morell (1) proposed a method for determining the peroxides in oxidized linseed oil by titrating the free iodine, liberated from a solution of potassium iodide in glacial acetic acid, with a standard sodium thiosulfate solution. Two years later, Yule and Wilson (2) estimated the peroxides in cracked gasoline by shaking it with an acidified solution of ferrous sulfate and ammonium thiocyanate in an acetone-water mixture and determining the ferric thiocyanate formed with a standard titanous chloride solution.

Several modifications of these two basic methods have been suggested. Using the iodometric method for determining the peroxide content of fats and oils, Stuffins and Weatheral (3) investigated the sources of error in the method and modified it by using either chloroform or carbon tetrachloride for the extraction of the liberated iodine. Risbey and Nisbet (4) compared the various methods in respect to the peroxide value of "motor spirits" and modified the original Yule and Wilson method by using zinc to reduce the trivalent iron to the bivalent state. The peroxide content of unsaturated organic compounds (5) has also been estimated colorimetrically by determining the amount of ferric thiocyanate with color standards.

Various sources of error exist in these methods. In the iodometric method and its modifications, there may occur the reabsorption of the liberated iodine by the materials and the oxidation of the reagents by air. Yule and Wilson (2), after an exhaustive study of the oxidation of ferrous sulfate by cracked gasoline, observed that there still remained a small amount of peroxides which reacted with potassium iodide. Nevertheless, the ferrous salt method and its modifications have been found to be more suitable and useful for comparative purposes even though complete reduction of all the peroxides present is not obtained.

* Received Nov. 6, 1947, from the Research Laboratories McKesson and Robbins, Inc., Bridgeport, Conn.

Whereas most of the materials investigated have been either cracked gasoline or oils, the amount of peroxides formed in mineral oil upon standing has never been thoroughly discussed or studied. Thus far, two quantitative procedures have been suggested for the deterioration of mineral oil. Assaf and Gladding (6), using the Grignard reagent, methyl magnesium iodide, claim that they have been able to estimate the amount of peroxides in mineral oil by means of the complicated Grignard reaction apparatus of Larson. Rae (7), on the other hand, measured the degree of decomposition of various paraffin oils, heated to 150°C., by determining the volume of 0.1 *N* potassium permanganate reduced.

Both of these methods are impractical as routine examinations. The procedure recommended by Assaf and Gladding requires the use of a complicated apparatus and skilled workers. With Rae's method, only poorly refined paraffin oils can be properly studied and compared.

The purpose of this paper is to introduce a simple and accurate method, which can be used for the quantitative determination of the peroxides formed in mineral oil.

It is an established fact that mineral oil upon prolonged standing or exposure to sunlight will develop a peculiar kerosene-like odor and taste. This is due to the formation of peroxides in the oil. The development of these peroxides is reported to be less likely in more highly purified oils than in inferior types.

Several qualitative methods have been suggested for determining the stability of mineral oil. Samples of mineral oil have been exposed to sunlight and to ultraviolet light in filma-sealed, flint bottles (8) and then observed for the possible presence of odors and flavors. Mineral oil stability has also been studied spectroscopically (9), and its absorption bands compared. Some investigators have studied the stability of oxidized paraffin oils by measuring the change in interfacial-tension (10) between a layer of 0.02 *N* sodium hydroxide and the oil.

Whereas the previously mentioned tests for determining the stability of mineral oils have been based upon certain physical factors, stability can also be determined chemically by the oxidation of the ferrous ion to the ferric ion by the peroxides present in heat-treated oils. The developing pink to red color can then be measured colorimetrically.

Several oil companies and individual investigators (11) have used a colorless test solution of ferrous sulfate, ammonium sulfocyanate, and sulfuric acid for the detection of peroxides. The oils were heated either under steam pressure or by direct heat. The development of a pink or red color in the test solution indicated the presence of peroxides, which constituted an objection with regard to stability.

A Peroxide Test method (12) has recently been proposed in which the expected shelf-life or stability of mineral oil is expressed in time units. The method consists of adding a colorless test solution of ferrous sulfate, ammonium thiocyanate, sulfuric acid, reduced iron, and iron wire to samples of oil heated in a 300° F. oil bath for various intervals and observing the developing color. The color is reported to vary from colorless to dark red, depending upon the amount of peroxides formed during the breakdown of the oil. The first sample exhibiting a pink coloration after shaking with the test solution is regarded as the Peroxide Test value, expressed in minutes.

EXPERIMENTAL

These facts and reactions have been used in developing the following quantitative method for the determination of peroxides formed in mineral oil. Samples of mineral oil were left in a 300° F. oil bath for one hour, and a colorless test solution, consisting of ferrous sulfate, ammonium thiocyanate, sulfuric acid, and reduced iron, was added to the heated oil. The developing pink or red color of the test solution was then measured colorimetrically in a Klett-Summerson photoelectric colorimeter.

Apparatus.—One-liter round-bottom flask and reflux condenser; test tubes $^3/_4 \times 6$ inches; constant temperature oil bath regulated at 300° F. ± 1° F.; glass-stoppered reaction flasks with greaseless stop cock, according to Connor and Straub's vitamin B_1 fluorometric method; centrifuge; Klett-Summerson photoelectric colorimeter with filter No. 56, having a mean transmission of 560 mμ, and special test-tube cells.

Materials.—Ferrous Sulfate C. P.; Ammonium Thiocyanate, C. P.; Iron by Hydrogen, Merck (reduced iron); Iron Wire No. 30, for standardization; Acetone U. S. P.; 3% Hydrogen Peroxide Solution.

Preparation of Test Solution.—In a round-bottom, wide-mouthed flask, 2 Gm. of ferrous sulfate is dissolved in 100 cc. of distilled water containing 2 cc. concentrated sulfuric acid. Two hundred cubic centimeters of acetone and 100 cc. of a 2% aqueous solution of ammonium thiocyanate are then added to the above solution. Iron powder 0.2 Gm. and 6 inches of No. 30 iron wire are finally added to reduce any trace of ferric ion present. The resulting mixture is refluxed on a steam bath until the red color of the solution has disappeared. This reduction process takes about two hours, it being necessary to continue refluxing to insure lack of color.

Fresh solution is prepared on the day of the test. It is possible, however, to preserve it in a colorless state by careful storage in a carbon dioxide atmosphere.

Since it is necessary to avoid any contact with air, a dispensing method has been devised. A syphon is passed into the upper end of the reflux condenser through a two-hole cork stopper. The second hole

is fitted with a glass stopcock, which serves as a vent. Carbon dioxide is admitted through the vent to inaugurate the flow of the solution, the pressure forcing the liquid into the syphon, at the end of which is attached a glass stopcock for the withdrawal of the solution.

Procedure.—Samples of mineral oil to be tested are placed in individual test tubes and immersed in a constant temperature oil bath at 300° F. ± 1° F. The tubes are removed after one hour's immersion in the oil bath and allowed to cool to room temperature. A 5-cc. sample of the heated mineral oil is pipetted into a glass-stoppered reaction flask which has been marked to indicate 5 cc. and 10 cc. filling. The air in the flask is replaced with CO_2, and 5 cc. of the colorless test solution is added. The flask is then tightly stoppered with the glass stopper, shaken by hand for one minute, and centrifuged for three minutes. The lower test solution layer which will then vary in color from pink to dark red is transferred quantitatively by means of the greaseless stopcock from the reaction flask to a cork-stoppered special Klett test tube cell, in which the air has also been replaced with CO_2. As a control, 5 cc. of unheated oil is treated similarly and run parallel with the heated sample. The test-tube cells, containing the sample and control, are then placed as soon as possible in the Klett colorimeter, and the amount of pink or red color formed is measured using the Klett filter No. 56. The difference in the scale readings between the unheated and heated oil samples gives the reading for the oxidation of ferrous thiocyanate in the test solution by the peroxides in 5 cc. of heated oil.

Prior to the above-described procedure, the Klett photoelectric colorimeter is adjusted until a zero reading is obtained with the colorless test solution and then standardized with various concentrations of hydrogen peroxide by measuring the amount of color corresponding to various quantities of peroxide. A freshly prepared 3% hydrogen peroxide solution is diluted 1–100, and its peroxide content is determined according to the U. S. P. assay method with 0.01 *N* potassium permanganate. Extremely small volumes of this diluted peroxide solution, having a concentration range of 1×10^{-6} to 1×10^{-4}, are added to 5-cc. portions of the colorless test solution in cork-stoppered Klett test-tube cells, and the amount of pink or red color formed is measured in the colorimeter.

Hydrogen peroxide concentrations are plotted against scale readings, and Fig. 1 represents the standardization curve obtained with increasing concentrations of peroxide. This graph facilitates the determination of the amount of peroxides in any 5-cc. sample of heated mineral oil by evaluating the concentration corresponding to the difference in scale readings between the control and sample.

It is essential that this standardization of the colorimeter be repeated on the day on which the test is to be carried out to correct any deviation in the colorimeter and possible changes in the standard peroxide solution.

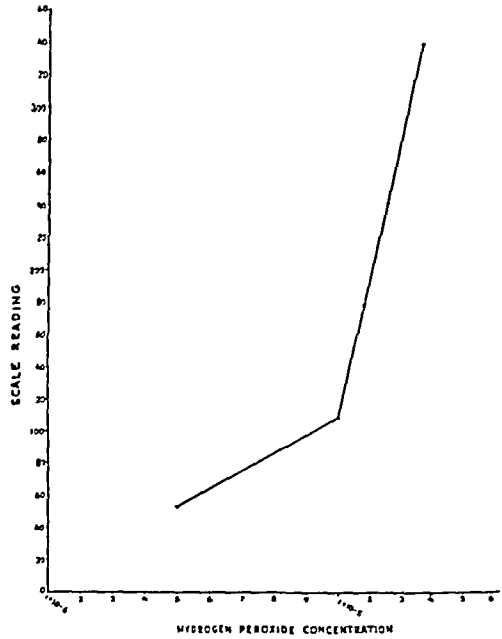

Fig. 1.—Standardization curve showing relationship of scale readings to hydrogen peroxide concentrations.

DISCUSSION

The described procedure makes possible not only the evaluation of the peroxides in mineral oil, but also the comparison of oils which, with the known methods of testing, would appear to have similar stability and quality.

Samples of mineral oil of several brands were tested accordingly. Table I summarizes the comparison of the Peroxide Values of the oils with their Peroxide Tests. The Peroxide Value is a quantitative expression for the amount of hydrogen peroxide formed in one liter of mineral oil, heated for one hour at 300° F. The Peroxide Test, on the other hand, is a qualitative test in which minutes of heating and detection of the degree of peroxides formed provide a means for establishing the stability of a mineral oil for various lengths of time.

TABLE I.—COMPARISON OF PEROXIDE VALUES WITH PEROXIDE TESTS OF MINERAL OILS

Sample of Various Brands	Peroxide Values, Grams of Hydrogen Peroxide per Liter of Heated Oil	Peroxide Test, Min.
A	0.00086	35
B	0.00116	30
C	0.00122	30
D	0.0012	22
E	0.005	15
F	0.0098	15
G	0.009	12
H	0.0116	12
I	0.0118	10
J	0.0182	10

The Peroxide Value reported for each sample represents the average of at least three separate determinations, which never varied more than ±3%.

The results obtained confirm and substantiate what has already been reported, namely, that an oil which develops peroxides after two minutes' heating is considered unstable and of poor shelf-life. Moreover, an oil which gives a colorless test with the ferrous thiocyanate solution after being heated twenty minutes or longer can be regarded stable and of very good quality (12).

The accuracy and reproducibility of the colorimetric method presented have been studied by means of dilute solutions of hydrogen peroxide of known concentration and by means of pure preparations of succinyl peroxide. For example, the concentration of hydrogen peroxide solution as given by the standard potassium permanganate method was 0.0036 mole per liter, while the colorimetric procedure gave 0.0037 mole per liter. The succinyl peroxide was prepared by the method of Clover and Houghton (13) and was recrystallized twice from acetone. The calculated weight per cent of active oxygen in succinyl peroxide is 6.83%; obtained by the colorimetric procedure 6.9%.

This suggested peroxide method offers a means of determining a new constant or property for both medicinal mineral oils and oils used in pharmaceutical and cosmetic preparations. Measurement of the amount of peroxides and the expected stability of different oils is made possible.

In the pharmaceutical use of mineral oil, it is of the utmost importance that careful consideration be given to the factor of stability. The values obtained by the above-described procedure offer themselves as a yardstick in this respect. Oils having a hydrogen peroxide content less than 0.005 gram per liter after heating for one hour at 300° F. will be of a high purity and stability and, therefore, suitable for prolonged medicinal use.

This quantitative method may also be used for determining accurately the amount of peroxides in other types of oil and may facilitate the evaluation of the effectiveness of antioxidants in those oils and preparations that readily turn rancid.

SUMMARY

A simple and accurate quantitative method is proposed for the determination of peroxides formed in mineral oil.

This colorimetric method may be used for estimating the peroxide content of other types of oil, such as cod-liver oil or vegetable oil, and also for evaluating the effectiveness of antioxidants.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

N-Methylhydroxylamine
Galactoflavin
2-Hydroxyundecylenic acid
2-Hydroxycaprylic acid
Avidin
Dihydrodypnone
Auxin A and B
Methionic acid
Coumarin-3-carboxylic acid
Urobilin
Dihydro-γ-benzopyrone
8-Methoxydihydro-γ-benzopyrone
Fluoromalonic acid
Chloromalonic acid
Dibenzofurane-3-sulfonic acid
2-(m-Hydroxyphenyl)ethylamine
N-Methyldiiodochelidamic acid
Thiophosgene

Magnesium Stearate. I. A Comparative Study of Commercial Samples*

By MARION L. LIEN† and C. E. MILLER‡

A comparative study of ten commercial samples of magnesium stearate involving physical and chemical tests has been made. The variable results obtained indicate that the samples were not identical in composition.

INCREASED PRODUCTION of commercial grades of magnesium stearate has been noted during the past five years. A survey of the literature disclosed the fact that no published comparative study of commercial grades of this chemical has been made. The importance of magnesium stearate to the cosmetic and paint industries, together with the lack of data concerning the relative compositions of available grades, made it of definite interest to conduct a comparative study. It is the purpose of this paper to present data obtained from such a study. Ten commercial samples were obtained from industry for this investigation and were numbered from one to ten inclusive.

Experimental

Analysis of Magnesium Stearate.—The several samples were characterized by studying their physical appearance, softening point, melting point, moisture, solubility in acetone and benzene, total ash, and magnesium content. The results of these determinations are summarized in the following tables.

TABLE I.—CHEMICAL AND PHYSICAL COMPARISON OF MAGNESIUM STEARATE

Sample	Softening Point, °C.	Melting Point °C.	Moisture, %[a]	Total Ash as MgO, %[b]
1	123	172–179	3.49	10.63
2	104	138–145	5.13	8.24
3	126	170–178	3.25	8.37
4	127	169–178	3.13	8.75
5	112	145–151	3.53	8.99
6	128	169–178	2.88	8.06
7	115	139–142	2.49	7.90
8	121	169–177	3.22	10.04
9	111	144–150	4.37	8.77
10	132	168–174	3.17	9.20

[a] Covered dishes used during weighing.
[b] Reported on a moisture-free basis.

* Received Jan. 5, 1948, from the School of Pharmacy, North Dakota Agricultural College, Fargo, N. D.
† Abstracted from a thesis submitted in partial fulfillment [illegible] for the degree of Master of Science in [illegible] Marion L. Lien, American [illegible] Education Fellow.
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It is evident from Table I that the magnesium stearate samples were not of identical composition. In the determination of the melting point it was noted in each case that the column of sample in the melting-point tube appeared to shrink or soften well below the melting-point range but not uniformly for the various samples. This phenomenon is reported under the heading of softening point. The melting-point bath was cooled to approximately 50° after each determination before again being used.

The theoretical amount of MgO that can be formed by the thermal decomposition of magnesium stearate, 6.82%, is less than the observed values, which is indicative of unreacted MgO or $Mg(OH)_2$. All of the samples were white in color, unctuous to the touch, and in a very fine state of particle size. Although pure magnesium stearate is reported to be soluble in acetone and in benzene (1), the samples studied were found to be incompletely soluble in these solvents, which is considered further proof that unreacted MgO or $Mg(OH)_2$ is present.

The pyrophosphate method used was essentially that of Kolthoff and Sandell (2), modified to make it applicable to magnesium stearate. Approximately 1.5-Gm. samples were ignited in nickel crucibles to constant weight and the resulting ash dissolved in 1:1 HCl. The magnesium was then precipitated in the usual manner as magnesium ammonium phosphate, collected on tared Gooch crucibles, washed free of chlorides and ignited to magnesium pyrophosphate. The above results are the average of duplicate determinations, expressed on a moisture-free basis.

Goldstein (3) determined magnesium by precipitating a solution of the magnesium salt with 8-hydroxyquinoline, thereby obtaining a yellow precipitate of magnesium hydroxyquinolate. This method, somewhat modified by Martin and Green (4), is the basis of assay for magnesium in Magnesium Citrate Solution. Application of this method to magnesium stearate samples was accomplished by liberating the magnesium from the soaps by acid hydrolysis. The resulting filtrates were made up to volume and analyzed. Twenty-five-cc. aliquots of the filtrate were precipitated as the 8-hydroxyquinolate, filtered, and dried to constant weight. The above results are the average of duplicate determinations, expressed on a moisture-free basis.

Analysis of Liberated Fatty Acids.—Because of the variances shown by the several samples in the analysis of magnesium stearate, it was believed that a study of the acidic portion of the soap would yield interesting and valuable information. Accordingly one hundred-gram quantities of the soaps were hydrolyzed with dilute sulfuric acid, and after purification the liberated fatty acids were evaluated by appropriate methods. The results of this part of the investigation are given in Table III.

The physical appearance of the liberated fatty acids both at room temperature and in the liquid

TABLE II.—PER CENT MAGNESIUM IN MAGNESIUM STEARATE

Sample	Mg, % Method A[a]	Mg, % Method B[b]	Sample	Mg, % Method A[a]	Mg, % Method B[b]
1	4.88	4.40	6	5.17	4.44
2	4.68	4.25	7	4.84	4.03
3	4.72	4.53	8	4.94	4.64
4	4.50	4.00	9	4.77	3.99
5	4.62	4.43	10	4.66	4.60

[a] Method A—pyrophosphate method.
[b] Method B—8-hydroxyquinolate method.

TABLE III.—COMPARATIVE ANALYSIS OF LIBERATED FATTY ACIDS

Sample	M. P., °C.	Titer, °C.	$n_D^{71.5}$	Iodine Value	Acid Value	Sp. Gr. 97° C.	Mol. Wt.
1	64.0	57.0	1.4348	1.25	180.6	0.8636	386
2	62.5	58.5	1.4331	4.00	196.7	0.8638	340
3	67.5	58.0	1.4351	1.76	170.9	0.8649	426
4	66.5	60.0	1.4364	3.23	167.2	0.8681	359
5	64.0	56.5	1.4332	3.21	189.9	0.8619	449
6	66.5	56.0	1.4358	2.43	166.8	0.8677	420
7	57.0	55.0	1.4324	3.70	198.4	0.8641	368
8	68.5	63.5	1.4352	2.44	167.5	0.8661	455
9	65.0	61.0	1.4328	4.59	196.4	9.8627	375
10	66.0	59.5	1.4352	2.63	158.1	0.8725	472

state was comparable to Eastman Kodak Company, white label Stearic Acid. The melting points reported were determined by the class II method of the U. S. P. The refractive index was determined in the usual manner, a circulating constant temperature bath set at 75° was satisfactory to maintain the reported temperature. The Rast Camphor Method (5) was used for determining the molecular weights. A future paper will discuss the distillation analysis of the several commercial samples.

SUMMARY AND CONCLUSIONS

1. Ten commercial samples of magnesium stearate have been compared by physical and chemical means.

2. Variations observed in melting points, solubility, total ash, and moisture content indicated that the samples were not of uniform composition.

3. Variable results by both methods for the determination of magnesium are in accord with the observations that commercial samples of magnesium stearate are mixtures.

4. The 8-hydroxyquinolate method for determining magnesium is applicable with certain modifications to the analysis of commercial samples of magnesium stearate.

5. The somewhat lower values found by the 8-hydroxyquinolate method may be due to solubility of the magnesium hydroxyquinolate in the precipitating media.

6. Further evidence of the variable composition of the samples was demonstrated in the analytical results observed in evaluating the liberated fatty acids.

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Estimation of Some Official Salts of Weak Organic Acids by Titration in a Nonaqueous Medium*

By MELVIN W. GREEN

A study was made of the potentiometric titration of a number of official salts of weak organic acids, notably sodium benzoate, sodium acetate, sodium propionate, barbital sodium, phenobarbital sodium, sulfathiazole sodium and sulfadiazine sodium, using a solvent mixture of propylene glycol and isopropyl alcohol and perchloric acid in the same solvent as a titrant. The results were compared with results obtained by the empirical use of indicators. In some instances when the potentiometric end point, determined with a Fisher titrimeter, gave low results, the empirical use of methyl orange or methyl red gave satisfactory results.

MOST ACIDIMETRIC and alkalimetric methods of analysis are based upon the older classical laws of the dissociation of acids and bases. Since most of the reactions take place in aqueous solutions or solutions predominantly aqueous there is no reason for not accepting the classical theories as they explain all of the phenomena taking place which need explanation.

In 1923, Bronsted and Lowry, working independently, developed a theory that acids should be defined as substances having a tendency to lose a proton (H^+) and bases as substances having a tendency to gain a proton. Since it is unlikely that protons ever exist free in solution, the acidic or basic properties are never seen independent of the solvent and indeed the solvent itself must exist as a proton donor or acceptor in order for dissociation to take place, e.g., a solution of an acid in petroleum benzin shows no conductivity.

If the protogenic or protophilic property of the solvent plays a role in the dissociation of an acid or base, it then follows that a substance may have its properties materially altered by a change in solvent. Such is indeed the case, and to cite a somewhat extreme case, nitric acid actually behaves as a base when dissolved in strong hydrogen fluoride solution where the following equilibrium is set up.

$$\underset{\text{base}}{HA} + \underset{\text{acid (solvent)}}{HF} \rightleftarrows \underset{\text{acid}}{H_2A^+} + \underset{\text{base}}{F^-}$$

The weaker an acid in aqueous solution the more likely it is to be a proton acceptor; hence dissolved in a protogenic solvent the more apt it is to behave like a strong base. The strength of bases is also influenced by the acidic or basic character of the solvent. An amine will thus behave as a very weak base in ammonia, a moderate base in water and as a very strong base in acetic acid (1, 2).

These facts have led several workers to carry on titrations in media other than water. Work has been somewhat slow from a practical viewpoint because the scale of acidity still has not been thermodynamically established and the value for the liquid junction potential is unknown in nonaqueous solutions. This brings about the very practical point of indicator selection for one does not know either the true *p*H of the end point of an acid-base titration, or the *p*H range of indicators under these conditions.

A further real handicap has been the limited solubility of many substances to be titrated. For example, although the sodium salts of most weak acids behave as strong bases when dissolved in alcohol, many of these salts are only moderately alcohol-soluble. Sodium benzoate, for example, is soluble 1 in 2, in water, but only 1 in 75, in alcohol; sodium propionate is soluble 1 in 1, in water, but only 1 in 24, in alcohol. Some of the other solvents are even poorer in this respect.

In 1946, Palit (3) showed that advantage could be taken of the solvating properties of the glycols on such salts, the $R.COO^-$ ion reacting with the glycol by hydrogen bonding to form a complete solution. If R is large enough, however, solution cannot be effected without the addition of a better hydrocarbon solvent such as isopropyl alcohol or butyl alcohol. Furthermore, it is difficult to titrate in a pure glycol medium because of the high viscosity of the glycol. By the use of 0.1 *N* to 0.2 *N* perchloric acid in a mixture of equal parts of propylene glycol or ethylene glycol and isopropyl alcohol, Palit was able to titrate such salts as sodium acetate and sodium propionate using either an indicator such as methyl orange (the choice of indicator is empiric) or a potentiometric end point in terms of apparent *p*H.

We set out to try to duplicate Palit's work and

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to explore the possibility of titrating such compounds as the sodium barbiturates and the sodium sulfonamides which are not carboxylic salts.

EXPERIMENTAL

The 0.1 *N* to 0.2 *N* perchloric acid was prepared by mixing approximately the correct quantity of 70% perchloric acid with a sufficient volume of equal parts propylene glycol and isopropyl alcohol. The small quantity of water in the 70% perchloric acid was the only amount of water in the entire system. This acid was standardized against 0.1 *N* alcoholic potassium hydroxide which in turn had been freshly standardized against pure potassium acid phthalate using methyl red as the indicator.

The salt or sodium compound was "dissolved" in a mixture of equal parts by volume of propylene glycol and isopropyl alcohol. At times solution was not complete but when the acid liberated (acetic, propionic, barbital, etc,) was sufficiently soluble in the mixed solvent to render solution complete when a fourth to a half of the reaction was completed, the method was considered to be satisfactory.

In nearly all cases the solution was stirred by a mechanical stirrer during the titration. Adequate agitation is more important in such titrations than in simple aqueous solutions for the viscosity of the solutions tends to make the reaction sluggish. Of equal importance is to wait at least one minute after adding titrant from the burette to obviate drainage error due to the viscosity of the solution.

In the potentiometric titrations, a Beckman *p*H meter with the conventional calomel and glass electrodes was used in the earlier studies. Later a Fisher titrimeter which employs the same electrode system, but has the additional advantage of a cathode tube (magic eye) indicator, was used. In the former case hand stirring was used.

In the indicator method, methyl orange, methyl red and, in a few instances, thymol blue, were used. It is worthy of note that these indicators gave much brighter colors in the mixed solvent but methyl orange still gave some of the traditional difficulty in ascertaining the exact end point. In almost every instance the indicator method used more titrant than the potentiometric method, i.e., the indicator gave a false end point beyond the true potentiometric end point. In some instances, as for example barbital sodium, this error actually enables one to obtain substantially the correct value for the analysis.

In Table I it may be seen that fairly good results were obtained in most of the salts determined, but that there is some tendency for the results to be high. Sodium propionate gave somewhat low results potentiometrically, however, as did sodium benzoate when the titrimeter was used. The discrepancy between the results for sodium benzoate using the *p*H meter and those using the titrimeter is difficult to explain unless with hand stirring the salt was overtitrated.

In Table II it may be seen that pentobarbital sodium gave equally low results with all three methods and barbital sodium gave good results when a colorimetric end point was chosen but poor results on either potentiometer.

From Table III it may be seen that sulfadiazine sodium gave very erratic results under all conditions. This can probably be attributed to the relative insolubility of both the sodium salt and the acid form of the drug in the medium employed. Sulfathiazole sodium, on the other hand, reacted very favorably.

The author considers this paper to be preliminary, for while it was shown that the method has possibilities it is believed that there are certain factors such as modification of solvent, speed of titrating, and indicator choice which need further study.

TABLE I.—NONAQUEOUS TITRATION OF SODIUM SALTS OF WEAK ACIDS

Salt	End Point	Recovery by Nonaqueous Titration %	Recovery by Official Method, %
Sodium Acetate, N. F.	Potentiometric	100.00	99.93
		100.24	99.87
Sodium Propionate, N. F.	Potentiometric	98.2	99.5
		98.8	99.2
	Colorimetric	99.1	99.3
Sodium Benzoate, U. S. P.	Potentiometric	99.73[a]	98.95
			99.24
		97.27[b]	99.18
	Colorimetric	100.6	
		99.64	
Sodium Salicylate, U. S. P.	Potentiometric	100.23	99.52
	Colorimetric	100.23	99.60

[a] *p*H meter.
[b] Fisher titrimeter.

SUMMARY

1. Using 0.1 *N* to 0.2 *N* perchloric acid in a mixture of equal parts of propylene glycol and isopropyl alcohol certain sodium compounds have

TABLE II.—NONAQUEOUS TITRATION OF SODIUM BARBITURATES

Compound	End Point	Recovery by Nonaqueous Titration, %	Recovery by Official Method, %
Barbital Sodium, U. S. P.	Potentiometric	95.66[a]	
		96.40[b]	
			99.32
			99.15
	Colorimetric	99.1	
		99.1	
Phenobarbital Sodium, U. S. P.	Potentiometric	100.2[a]	
		99.15[b]	
			98.93
			99.24
	Colorimetric	100.7	
		100.3	
Pentobarbital Sodium, U. S. P.	Potentiometric	97.57[a]	
		96.67[b]	
			99.27
			99.46
	Colorimetric	97.62	
		97.48	

[a] pH meter.
[b] Fisher titrimeter.

TABLE III.—NONAQUEOUS TITRATION OF SODIUM SULFONAMIDES

Compound	End Point	Recovery by Nonaqueous Titration, %	Recovery by Official Method, %
Sulfadiazine Sodium, U. S. P.	Potentiometric	101.5[a]	
		93.4[b]	
		88.8[b]	
			99.68
			99.73
	Colorimetric	101.6	
		103.1	
		100.1	
Sulfathiazole Sodium, U. S. P.	Potentiometric	99.33[a]	
		100.9[a]	
		99.3[b]	
			99.08
			99.14
	Colorimetric	99.42	
		99.52	

[a] pH meter.
[b] Fisher titrimeter.

been titrated using indicator dyes and potentiometers to determine the end point.

2. Sodium acetate, sodium benzoate, sodium salicylate, phenobarbital sodium, and sulfathiazole sodium gave favorable results. Barbital sodium gave low results with a potentiometric titration but good results when an indicator was used. Pentobarbital sodium gave relatively low results by all methods. Sulfadiazine sodium cannot be titrated in this manner, at least without considerable modification of the method.

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An Improved Procedure for the Extraction of Phlorizin*

By GEORGE D. CURTS†

A method is given whereby phlorizin is obtained, from fresh apple root bark, as a pearly-white, lustrous solid.

PHLORIZIN is a glucoside found in the root bark of various species of apples in the amount of about 0.2%. It is known to lower the threshold of sugar retention by the kidneys, thus inducing a glycosuria. Continued interest (1) in the pharmacological properties of phlorizin has encouraged the search for improvements in the extraction procedure.

EXPERIMENTAL

The fresh root bark is collected and shredded in a hammer mill very soon thereafter, although it may be stored in a refrigerated room for a day or so if necessary. The bark shreds very well at a 1/4-inch mesh setting. After shredding, the bark is immersed in boiling water and steeped for one hour in a standard manner (2), steam being introduced to maintain the boiling temperature. The resulting mash is strained and the steeping process repeated. The material is again strained and the combined strained liquids allowed to stand for twenty-four hours. With some species, impure phlorizin will separate from the menstruum at this time.

If phlorizin does not separate, the solution is evaporated to dryness under reduced pressure, or, if preferred, under normal pressure with the addition of calcium or magnesium carbonate when nearly dry. The residue is then extracted with hot ethyl acetate and this solution concentrated.

Impure phlorizin may be precipitated by the addition of chloroform, but interfering resinous material is also precipitated. This material may be removed by first precipitating with two volumes of ether. The phlorizin remains in solution and is obtained by the addition of one volume of chloroform.

The phlorizin precipitate is then purified by treatment with activated charcoal in hot aqueous solution, or by alternate aqueous and ethyl acetate-chloroform crystallization.

Phlorizin obtained by this method is a pearly-white, lustrous solid. The hydrous form melts at 114° (unc.) and the anhydrous at 158° (unc.)

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Diethylaminoethyl Dialkylacetates*

By RAMAN C. AMIN, WALTER H. HARTUNG, and CLIFFORD W. CHAPMAN

The synthesis is reported of a series of diethylaminoethyl esters of dialkylacetic acids in which the carbon content varied from six to eight atoms. Four esters of alpha-bromo acids were also prepared. All compounds described were tested for surface anesthetic action but none was effective.

PROCAINE analogs in which the acyl component of the molecule is aliphatic are not new. Diethylaminoethyl acetate shows no anesthetic action (1). The corresponding esters of the higher aliphatic, straight-chain acids exhibit some activity; their effectiveness is reported to be proportional to their molecular weight (2, 3). Unsaturation in the straight carbon chain of the acid apparently modifies the pharmacological properties but little (2), although the esters of acrylic acid derivatives, $RCH{=}CHCOOCH_2CH_2N(C_2H_5)_2$ are distinctly, though weakly, active (4).

Among these procaine analogs little or no mention is made of the esters derived from

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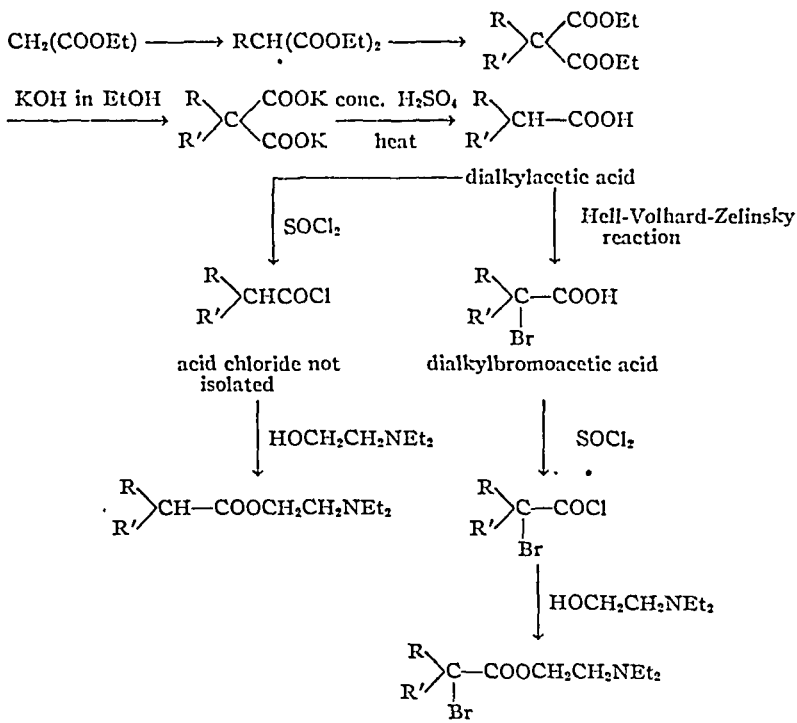
branched-chain aliphatic acids. In view of the fact that branched-chain compounds frequently differ in pharmacological properties, either quantitatively or qualitatively, from their normal chain isomers, it was felt that the dialkylaminoalkanol esters of branched-chain acids merited investigation. Accordingly a series of diethylaminoethyl esters of dialkylacetic acids were prepared in which the carbon content varied from six to eight atoms. Since the presence of halogen in an organic molecule increases lipoid solubility, four esters of α-bromo acids were also prepared.

EXPERIMENTAL

The compounds were prepared according to the sequence of reactions shown below:



The acids obtained are listed with pertinent data in Table I.

The acids were allowed to react with thionyl chloride. The resulting acid chloride was not isolated, but was dissolved in dry benzene, treated directly with a solution of diethylaminoethanol in benzene, and the mixture refluxed for one hour. The organic mixture was washed with aqueous potassium hydroxide solution and the organic layer was then dried over anhydrous sodium sulfate before distillation. The desired ester was collected at the proper point. A typical synthesis, that of diethylaminoethyl *n*-butylethylacetate, is described below.

Diethylaminoethyl *n*-Butylethylacetate.—In a 500-cc., 2-neck, round-bottom flask, equipped with a dropping funnel and reflux condenser which was connected to an absorption trap for hydrogen chloride, was placed 21.6 Gm. (0.15 mole) 2-ethylhexanoic acid (*n*-butylethylacetic acid). This was heated on a water bath, and during the course of a half hour 24 Gm. (0.2 mole) of thionyl chloride was added through the dropping funnel. The mixture was heated for about three hours until all the acid was dissolved; excess of thionyl chloride was removed by distillation. The acid chloride was dissolved in 40 cc. dry benzene. The two dropping funnels containing this solution and 19.92 Gm. (0.17 mole)

In order to evaluate the local anesthetic activity of these esters, 2 per cent solutions of the compounds in sterilized water having the *p*H adjusted between 6 and 7 were tested by the rabbit's cornea method described by Schmitz (5) and Harry (6) and their co-workers. An unbuffered 1 per cent aqueous solution of cocaine was used as the control.

None of the esters prepared showed any anesthetic activity in 2 per cent solution; when the concentration was increased to 6 per cent there was still no apparent anesthesia, but severe irritation and inflammation were produced.

TABLE I.—DIALKYLACETIC ACIDS

RR'CHCOOH R	R'	B. P. in °C. at 18 Mm.	Density d^{33}_{33}	Formula	Neutralization Equivalent Calcd., %	Found, %
Ethyl[7]	Ethyl	99–101	0.9237	$C_6H_{12}O_2$	116.13	117.42
n-Propyl[8]	Ethyl	105–107	0.9361	$C_7H_{14}O_2$	130.15	131.43
Isopropyl[9]	Ethyl	110–112	0.9076	$C_7H_{14}O_2$	130.15	132.15
n-Butyl[10]	Ethyl	118–120	0.9029	$C_8H_{16}O_2$	144.17	141.66
Isobutyl[11]	Ethyl	120–122	0.9023	$C_8H_{16}O_2$	144.47	143.15
RR'CBr—COOH R	R'	Yield on Bromination, %				
n-Butyl[12]	H	51.76	136–138	1.2812	$C_6H_{11}O_2Br$	195.04	191.92
Isopropyl	Ethyl	33.4	123–125	1.0680	$C_7H_{13}O_2Br$	209.06	211.94
n-Butyl	Ethyl	31.2	145–148	1.2900	$C_8H_{15}O_2Br$	223.08	221.41
Isobutyl	Ethyl	48.1	127–129	1.0313	$C_8H_{15}O_2Br$	223.08	226.35

TABLE II.—DIETHYLAMINOETHYL DIALKYLACETATES

RR'CH—COOCH$_2$-CH$_2$N(C$_2$H$_5$)$_2$ R	R'	B. P. in °C. at 18 Mm.	Yield, %	d^{32}_{32}	n^{31}_{D}	Formula	Nitrogen Analysis (Kjeldahl) Calcd., %	Found, %
n-Propyl[3]	H	103–105	53.4	0.8862	1.4299	$C_{10}H_{21}O_2N$	7.48	7.62; 7.64
n-Butyl[3]	H	131–133	63.6	0.8937	1.4329	$C_{12}H_{25}O_2N$	6.51	6.73; 6.71
Ethyl	Ethyl	109–111	53.6	0.8858	1.4294	$C_{12}H_{25}O_2N$	6.51	6.43; 6.42
n-Propyl[3]	Ethyl	115–118	46.5	0.8946	1.4347	$C_{13}H_{27}O_2N$	6.11	5.87; 5.88
Isopropyl	Ethyl	120–122	43.6	0.8938	1.4339	$C_{13}H_{27}O_2N$	6.11	6.34; 6.32
n-Butyl[2]	Ethyl	127–129	66.6	0.8965	1.4366	$C_{14}H_{29}O_2N$	5.76	5.91; 5.89
Isobutyl	Ethyl	133–135	43.2	0.8960	1.4360	$C_{14}H_{29}O_2N$	5.76	5.61; 5.59
RR'CBr—COOCH$_2$CH$_2$N(C$_2$H$_5$)$_2$ R	R'							
n-Butyl	H	119–121	25.0	...	1.4442	$C_{12}H_{24}BrO_2N$	4.76	4.61; 4.58
Isopropyl	Ethyl	127–129	9.7	...	1.4478	$C_{13}H_{27}BrO_2N$	4.54	4.41; 4.44
n-Butyl	Ethyl	122–124	9.3	...	1.4476	$C_{14}H_{29}BrO_2N$	4.34	4.27; 4.29
Isobutyl	Ethyl	135–137	12.9	...	1.4461	$C_{14}H_{29}BrO_2N$	4.34	4.40; 4.41

of diethylaminoethanol in 40 cc. benzene, respectively, were fitted to a 3-neck round-bottom flask equipped with a mechanical stirrer. The two solutions were added dropwise and simultaneously with stirring to 40 cc. of benzene in the flask. The mixture was refluxed for one hour, then poured into 200 cc. of water, and made alkaline with 10% KOH solution. The benzene layer was separated and dried over anhydrous sodium sulfate and the benzene was removed by distillation under atmospheric pressure. The residue was distilled under reduced pressure to obtain a yield of 23 Gm. (66.6% of the theoretical amount) of diethylaminoethyl 2-ethylhexanoate, b. p. 127–129° 18 mm.

SUMMARY

(1) Nine branched-chain aliphatic acids were prepared, of which α-bromo-iso-propylethylacetic acid, α-bromo-*n*-butylethylacetic acid, and α-bromo-iso-butylethylacetic acid are not described in the available literature.

(2) Seven diethylaminoethyl dialkylacetates and four diethylaminoethyl α-bromodialkylacetates were prepared; of these the hydrochlorides of diethylaminoethyl *n*-butyrate, caproate, and heptoate; and diethylaminoethyl *n*-octoate have been previously described.

(3) The results of the pharmacological studies of these esters indicate that none possesses any surface anesthetic activity.

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Detoxification of Pyrogens by Hydrogen Peroxide in Some U. S. P. Injections*

By ABRAHAM TAUB† and FANCHON HART‡,§

The detoxification of pyrogens by boiling with a low concentration of hydrogen peroxide has been found applicable to water for injection, normal saline solution, and dextrose and sodium chloride injection. Excess peroxide is decomposed with manganese dioxide, or in the case of dextrose-saline, by activated charcoal. Selas candle filtration removes the decomposing agent. The method produces no significant alteration in the composition of the injection fluids.

THE REMOVAL of bacterial pyrogens from parenteral solutions has been the subject of considerable attention since Hort and Penfold (1) first showed that these fever-producing substances could not be eliminated by conventional sterilization techniques. Adsorption filtration through specially prepared asbestos pads has been the most widely accepted procedure for pyrogen removal from solutions of crystalloidal substances. That this method has not proved universally acceptable is indicated by the modifications which have been recommended. Francke and Reese (2) have advocated charcoal adsorption and filtration, followed by Seitz pad filtration. Simpler adsorption techniques using activated charcoal have been utilized for the removal of limited amounts of pyrogen from solutions containing nonadsorbable active ingredients (3). More recently, Smith and Pennell (4) have suggested the removal of pyrogen from plasma protein solutions by digestion with relatively large quantities (3 to 24 per cent) of an ion exchange agent (Decalso), followed by filtration through improved Seitz asbestos pads.

Chemical detoxification of pyrogen has received but limited attention, primarily because of the destructive effect of reagents upon medicinal agents, and because of the difficulties of removing the excess reagent and its by-products. Co-Tui (5) has reported that pyrogen may be destroyed by boiling for one-half hour with 0.1 *N* HCl. Carter (6) has found dilute potassium permanganate solution useful in destroying pyrogen prior to distilling water in all-glass stills. The results of the researches of Robinson and Flusser (7), indicating the polysaccharide nature of pyrogen, suggest the possibility of destroying pyrogen activity by combined hydrolytic cleavage and oxidation.

Of the many available oxidizing agents, hydrogen peroxide would appear to be one of the most promising for depyrogenizing aqueous solutions, since water is its only end product. Obviously it may be used only where, in the concentration employed, it exerts no destructive effect upon the therapeutic or other ingredients of the parenteral solution. There is, however, a dearth of literature on this use of hydrogen peroxide. Hort and Penfold (8) reported in 1912 that centrifuged cells of *Eberthella typhosa*, when washed with hydrogen peroxide, lost their fever-producing effects when injected intravenously. Campbell and Cherkin (9), in a preliminary report of their investigation of plasma substitutes, observed that gelatin solution was rendered nonpyrogenic by boiling with 0.1 *M* H_2O_2 for two hours, or by autoclaving with 0.04 *M* H_2O_2 at 116° for twenty minutes. They did not, however, indicate the effect, if any, of the peroxide upon the gelatin. Nor did they indicate that any excess hydrogen peroxide present after boiling was removed. It should be noted that the liberation of oxygen by catalase when hydrogen peroxide is introduced intravenously may give rise to gas bubbles, if local oxygen solubility is exceeded, with possible fatality from embolism.

It is the purpose of the present study to evaluate hydrogen peroxide as a pyrogen detoxifier in some U. S. P. injections which are intended for intravenous infusion in relatively large volumes.

EXPERIMENTAL

The procedure employed consisted of preparing a concentrated pyrogenic broth and adjusting its potency to a specific number of minimum pyrogenic doses (*M. P. D.*) per 10 cc. injected per Kg. of body weight of rabbit. [Co-Tui (10) has defined the *M. P. D.* as the dose per Kg. of body weight which upon intravenous injection will cause a rise of 0.5 to 0.6° within four hours. Throughout this work the U. S. P. threshold of 0.6° within three hours has been used.] The standardized pyrogenic stock liquid was then added in increments up to 1000 *M. P. D.* per 10 cc. to Water for Injection, Normal Saline Solution, and Dextrose and Sodium Chloride Solution. The pyrogenic solutions were then treated

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with hydrogen peroxide in different concentrations and under varying conditions. The excess peroxide was then decomposed and the solutions filtered aseptically and injected into rabbits to determine their pyrogenic effect.

A Difco nutrient agar slant of *E. typhosa* (Type No. 6539 F. D. A., Hopkins Strain 26) was prepared, and after incubation for forty-eight hours the bacterial growth was washed off with Difco nutrient broth. The bacterial suspension was made up to one liter with broth and incubated at 37° for forty-eight hours. Based on plate count and nephelometric readings, the bacterial population was estimated at 4×10^8 per cc. This stock pyrogen solution was autoclaved at 15 lb. for fifteen minutes and checked for sterility. Dilutions ranging from 20 cc. per 100 cc. to 0.01 cc. per 100 cc. were made in sterile pyrogen-free saline solution, and the solutions filtered aseptically through Selas 015 porcelain filters (1.4 micron porosity). The solutions were injected intravenously in dosage of 10 cc. per Kg. in New Zealand rabbits weighing 1.5 to 2.65 Kg. The 0.01 cc./100 cc. dilution gave a maximum rise of 0.35°. The 0.02 cc./100 cc. dilution gave a maximum rise of 0.62°, representing 1 *M. P. D.* The highest concentration used, 20 cc. of pyrogen stock solution, representing 1000 *M. P. D.*, gave a maximum rise of 2.80°, and resulted in one fatality.

Water for Injection, Normal Saline Solution, and Dextrose and Sodium Chloride Injection (5% dextrose and 0.9% sodium chloride) were prepared and rendered pyrogen free by Ertel filtration, and autoclaved for fifteen minutes at 20 lb. for the water and normal saline, and for sixteen minutes at 11 lb. for the dextrose-saline. They were checked for sterility and freedom from pyrogens. Increments of stock pyrogen solution, corresponding to the amounts of *M. P. D.* indicated in Tables I and II, were added to the injection fluids. Treatment with hydrogen peroxide was carried out in one of three ways:

Method 1.—Using a reflux condenser bearing a sintered glass vent, the solution was boiled for one hour with H_2O_2, in concentrations ranging from 0.02% to 1%. After cooling to 90°, the residual peroxide was decomposed with small portions, 5×0.02 Gm., manganese dioxide (N. F. Reagent) per 100 cc. of solution, care being taken to avoid overfoaming during the first few minutes. The liquid was then boiled for five minutes to remove dissolved oxygen, and filtered aseptically through a Selas 02 candle (0.85 micron porosity) into a pyrogen-free container. Intravenous injections of 10 cc. per Kg. of body weight were made within an hour after preparation. A 30% H_2O_2 was used to minimize the volume change due to peroxide addition. Experi-

TABLE I.—DETOXIFICATION OF PYROGEN IN NORMAL SALINE SOLUTION

Expt.	Pyrogen Added before Treatment: Stock Pyrogen, Cc./100 Cc.	Pyrogen Added before Treatment: *M. P. D.*/Kg.	H_2O_2, %	Method of Treatment	MnO_2, Mg./100 Cc.	Maximum Temperature Rise, °C.	Average of Maximum Temperature Rises, °C.
A	1	50	0.5	3	..	1.15	1.01
B	1	50	0.1	1	..	0.35	0.30
C	1	50	0.02	2	0.5	0.58	0.55
D	2	100	1.0	3	..	2.05	1.78
E	2	100	1.0	2	10.0	0.75	0.73
F	2	100	0.1	2	0.5	0.45	0.45
G	5	250	1.0	2	0.5	0.20	0.10
H	5	250	0.5	2	10.0	1.00	0.70
I	5	250	0.2	2	0.5	0.50	0.48
J	5	250	0.1	2	0.5	0.45	0.40
K	5	250	0.02	1	..	1.00	0.72
L	5	250	0.02	2	0.5	0.80	0.72
M	20	1000	1.0	2	10.0	0.80	0.76
N	20	1000	0.1	2	10.0	2.50	2.35
O	20	1000	0.1	2	0.5	0.52	0.50
P	20	1000	0.1	2	0.5	0.30	0.20
Q	20	1000	0.1	1	..	0.44	0.42

TABLE II.—DETOXIFICATION OF PYROGEN IN DEXTROSE AND SODIUM CHLORIDE INJECTION

Expt.	Pyrogen Added before Treatment: Stock Pyrogen Cc./100 Cc.	Pyrogen Added before Treatment: *M. P. D.*/Kg.	H_2O_2, %	Method of Treatment	MnO_2, Mg./100 Cc.	Charcoal, Mg./100 Cc.	Maximum Temperature Rise, °C.	Average of Maximum Temperature Rises, °C.
A	5	250	1.0	3	..	...	1.50	1.47
B	5	250	None	1 hr. boil	5	...	1.30	1.20
C	5	250	0.1	1	..	...	0.55	0.42
D	5	250	0.1	2	5	...	0.24	0.10
E	5	250	0.1	2	..	100	0.24	0.18
F	20	1000	0.1	2	5	...	0.15	0.12
G	20	1000	0.1	2	..	100	0.31	0.20

ments were limited to one hour of boiling after preliminary tests showed that one-half hour was inadequate.

Method 2.—The twofold purpose of this method was to release oxygen continuously in minute quantities for the duration of boiling, and to reduce the amount of peroxide remaining to be decomposed after boiling. Therefore, small quantities of manganese dioxide, as indicated in Tables I and II, were added to the solutions prior to boiling. After one hour of boiling under reflux, any residual peroxide was decomposed by manganese dioxide (0.1 Gm. per 100 cc.), and the balance of the procedure followed as in Method 1.

Method 3.—The solution was allowed to stand for twelve hours with hydrogen peroxide at room temperature (20–25°) under a sintered glass vent. Then 0.1 Gm. manganese dioxide per 100 cc. was added cautiously in 0.02-Gm. increments, and after fifteen minutes, when most of the peroxide had been decomposed, the solution was brought to a boil under a reflux and heated for five to ten minutes to complete the peroxide decomposition and expel dissolved oxygen. The solution was then filtered through a Selas candle as in Method 1.

In all cases, the solutions were tested qualitatively for freedom from peroxide before being injected into the test animals.

In a few instances activated charcoal (Nuchar) was used to replace manganese dioxide as peroxide decomposing agent, for reasons to be explained below.

Table I shows the extent of pyrogen detoxification in normal saline solution. The results for injection water were found to parallel so closely those of the normal saline as to preclude the use of a separate table.

Experiment F, Table I, was repeated, using activated charcoal (0.1 Gm. per 100 cc.) in place of manganese dioxide. Upon injection this produced a maximum rise of 0.4° and an average maximum rise of 0.33°.

Table II shows the extent of detoxification of pyrogen in Dextrose-Saline Solution, manganese dioxide or activated charcoal serving as peroxide decomposing agents.

An examination of the data of both tables makes it readily apparent that hydrogen peroxide used at room temperature (Method 3) is not a satisfactory pyrogen detoxifier up to 1% concentration. Higher concentrations would not prove practical. Additional experiments carried out at room temperature showed that this method was not improved by providing for continuous evolution of oxygen by the addition of 0.5 to 50 mg. quantities of MnO_2 per 100 cc. of peroxide-treated injection fluids. On the other hand, boiling for an hour with peroxide (Method 2) in concentrations as low as 0.02% detoxified moderately large amounts of pyrogen (50 *M. P. D.* per 10 cc.) and 0.1% peroxide under the same conditions detoxified 1000 *M. P. D.* per 10 cc. injection fluid.

Although there are indications that Method 2 is slightly superior to Method 1, the limited number of experiments does not warrant any positive conclusions. However, the small amount of MnO_2 does provide smoother boiling, and reduces or eliminates the residual peroxide to be decomposed. In dealing with large volumes of parenteral solutions, too rapid addition of manganese dioxide at the end of boiling in Method 1 may cause copious frothing for a few minutes. It is desirable, therefore, that the residual peroxide not exceed a few hundredths of a percentum. Method 2 accomplishes this purpose.

On the other hand, too much MnO_2 during the hour of boiling with hydrogen peroxide, even 10 mg. per 100 cc. in the case of saline (Experiments E, H, M, and N, Table I), does not permit effective peroxide detoxifying action. That the manganese dioxide *per se*, in the absence of hydrogen peroxide, is ineffective at boiling temperature as a pyrogen detoxifier, can be seen from the control Experiment B, Table II.

Although MnO_2 proved satisfactory in decomposing residual peroxide in water and saline injections, it produced a slight discoloration in dextrose-saline. Therefore Nuchar was used in its place. Experiments E and G, Table II, show that it is effective, though higher concentrations than for MnO_2 are required. The experiments with MnO_2 were nevertheless carried out, and are included in Table II to permit proper evaluation of hydrogen peroxide as a pyrogen detoxifier and avoid introduction of an extraneous factor, namely, the adsorption of pyrogen by activated charcoal.

When the problem of detoxification of pyrogen by hydrogen peroxide was first approached, it became apparent that the removal of excess peroxide would have to be successfully solved if the method were to prove serviceable. A search of the literature and some preliminary experiments narrowed the field of suitable peroxide deactivating agents to two substances of practical interest, manganese dioxide and activated charcoal. Although both of these may decompose higher concentrations of hydrogen peroxide with explosive violence, the rate of peroxide decomposition at 0.1% H_2O_2 concentration is readily controlled. To determine suitable concentrations of manganese dioxide and charcoal, the experiments listed in Tables III and IV were conducted at the boiling point of water, using saline and dextrose-saline injections, respectively. Residual peroxide concentrations were determined by titration with standard potassium permanganate solution. Dextrose did not interfere in the dilution at which the titrations were carried out.

It is of interest to note that water and saline behave similarly with respect to the influence they exert upon manganese dioxide and activated charcoal as peroxide decomposing agents. The data also provide the reason why 10 mg. of MnO_2 per 100 cc. proved unsatisfactory in Experiments E, H, M, and N in Table I, since effective peroxide concentration does not last more than a few minutes at the boiling point in the presence of this quantity of MnO_2. Dextrose exerts a retarding effect on the decomposition of peroxide by both agents; therefore more is required of either to bring the peroxide level down to the concentration present in water or saline during the boiling process. About 5 to 10 mg. MnO_2 per 100 cc., or 100 to 200 mg. activated charcoal were optimum for the reagents used.

Experiments conducted at room temperature showed that in the absence of manganese dioxide or charcoal, after a twelve-hour digestion about 75% of the original concentration of hydrogen peroxide is retained in the water and saline injections, and over 90% is retained in the dextrose-saline, when an initial concentration of 0.1% H_2O_2. was used.

TABLE III.—RESIDUAL CONCENTRATIONS OF HYDROGEN PEROXIDE IN INJECTION WATER, NORMAL SALINE SOLUTION, AND DEXTROSE-SALINE INJECTION AFTER BOILING WITH MANGANESE DIOXIDE AND 0.1% HYDROGEN PEROXIDE

Time of Boiling, Min.	Percentage of Hydrogen Peroxide: Injection Water MnO_2: Mg./100 Cc.			Normal Saline Solution MnO_2: Mg./100 Cc.			Dextrose-Saline Injection MnO_2: Mg./100 Cc.		
	0	0.5	5.0	0	0.5	5.0	0	5.0	5.0
15	0.080	0.043	0.016	0.072	0.050	0.012	0.095	0.092	0.072
30	0.060	0.014	0.000	0.042	0.022	0.000	0.092	0.086	0.064
45	0.027	0.007	0.000	0.020	0.012	0.000	0.089	0.071	0.058
60	0.020	0.003	0.000	0.010	0.003	0.000	0.085	0.068	0.036

TABLE IV.—RESIDUAL CONCENTRATIONS OF HYDROGEN PEROXIDE IN INJECTION WATER, NORMAL SALINE SOLUTION, AND DEXTROSE-SALINE INJECTION AFTER BOILING WITH ACTIVATED CHARCOAL AND 0.1% HYDROGEN PEROXIDE

Time of Boiling, Min.	Percentage of Hydrogen Peroxide: Injection Water Charcoal: Mg./100 Cc.			Normal Saline Solution Charcoal: Mg./100 Cc.			Dextrose-Saline Solution Charcoal: Mg./100 Cc.		
	0	100	1000	0	100	1000	0	100	1000
15	0.080	0.056	0.006	0.072	0.046	0.005	0.095	0.066	0.017
30	0.062	0.044	0.000	0.042	0.030	0.000	0.092	0.058	0.000
45	0.027	0.022	0.000	0.020	0.014	0.000	0.089	0.048	0.000
60	0.020	0.017	0.000	0.010	0.008	0.000	0.085	0.040	0.000

It should be noted that the data in Tables III and IV apply to the particular deactivating reagents used in this study. Particle size and other variables may influence the rate of peroxide decomposition. Several grades of manganese dioxide tested did not produce significantly different results from those in the tables. However, activated charcoals differ considerably in properties, and preliminary tests of their rates of decomposing H_2O_2 should be made before applying them for this purpose.

All three injection fluids were examined after processing by Method 2. The *p*H of the water was 5.7 before treatment; it was 6.2 after treatment when charcoal was used, and 6.9 when MnO_2 was used. The rise appears due to CO_2 expulsion during boiling. The water after treatment met the U. S. P. permanganate test for limit of oxidizable substances. The total solids after treatment was 0.7 mg. per 100 cc. (U. S. P. limit is 1.0 mg.). It should be noted that since the U. S. P. permissible limit of acid-soluble substances in activated charcoal is 35 mg. per Gm., charcoal should be boiled with dilute hydrochloric acid and washed with water prior to use.

The *p*H of the saline solution changed only slightly, from 6.8 to 6.68 when manganese dioxide was used, and to 6.9 after charcoal treatment. The sodium chloride content was unaltered. In both saline solution and water, no traces of hydrogen peroxide or manganese ion were detected. The liquids were odorless and colorless.

Examination of the dextrose-saline after treatment with peroxide and manganese dioxide showed that the total acidity remained within U. S. P. limits for dextrose. The *p*H dropped from 6.86 to 5.9. When charcoal was used it dropped to *p*H 5.2. It is usual for the *p*H of dextrose solutions to drop after heat sterilization. A slight yellow color and a trace (0.5 p. p. m.) of manganese ion were found in the dextrose-saline after filtration. Therefore, manganese dioxide should be replaced by activated charcoal in the treatment of dextrose solutions. The charcoal yields a colorless filtrate.

The possibility of dextrose oxidation by the peroxide was next examined. Payne and Foster (11) have shown that when dextrose is treated with hydrogen peroxide, using 2 to 5 moles of peroxide per mole of dextrose, and heating for eighteen to twenty-four hours, about 50% to 80% is converted to gases, mainly CO_2, and about 1% to 40% is oxidized to aliphatic acids. However, such rigorous oxidation cannot be compared with an hour of boiling at a concentration of 0.1% H_2O_2. Polarimetric examination of the dextrose-saline solution after peroxide treatment showed only a slight drop in optical rotation, from 4.84° to 4.76° after the use of manganese dioxide, and from 4.84° to 4.81° when activated charcoal was used as peroxide decomposing agent. It is probable that a slight amount of gluconic acid or other carboxylic acid was formed which accounts for the slight drop in *p*H. However, it is known that a similar slight oxidation occurs even in the absence of H_2O_2, when dextrose solution is autoclaved in commercial practice. The trace of manganese ion found in the dextrose saline solution could be accounted for on the basis of the solvent action of the carboxylic acids on the manganese dioxide. By replacing the MnO_2 with activated charcoal, this problem is circumvented.

DISCUSSION

The peroxide method of detoxifying pyrogenic solutions is worthy of consideration where no oxidative changes are produced in the active ingredients, and where the requisite H_2O_2 concentration may be maintained during boiling. Peroxide concentrations of the order of 0.05% to 0.1% are desirable during the first fifteen minutes of boiling. Concentrations of 0.003% to 0.03% are desirable after an hour of boiling. It may also be noted that for water and saline, but not for dextrose solutions, boiling for two hours with an initial concentration of 0.1% H_2O_2 has been found in most instances to yield a peroxide-free solution, obviating the need of a peroxide decomposing agent.

The peroxide method, where applicable, offers

such advantages over adsorption filtration as freedom from asbestos fibers or haze in the filtrate, simplicity of cleaning and sterilizing the filter unit, capability of detoxifying greater amounts of pyrogen, and applicability to other than crystalloidal solutions.

SUMMARY

A method for detoxifying pyrogens in water, saline, and dextrose-saline solutions has been tested, using a calibrated pyrogen preparation derived from *E. typhosa*.

It has been found that boiling with 0.1% of hydrogen peroxide for one hour is capable of detoxifying 1000 minimum pyrogenic doses per 10 cc. in these injection fluids.

For the removal of residual peroxide, a limited quantity of manganese dioxide, during the boiling, is suggested for use in water or saline; purified activated charcoal, acid- and water-washed, should be used for this purpose for dextrose solutions. Filtration through an 02 Selas candle removes the peroxide decomposing agent and yields a particle-free solution.

No evidence has been found of significant change in the injection fluids subjected to this method of detoxifying pyrogen.

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The Applications of Radioactive Tracer Techniques to Pharmacy and Pharmaceutical Research*

By JOHN E. CHRISTIAN

A brief review of some of the applications of radioactive isotopes to pharmaceutical research is presented. Subsequent reports will describe experimental procedures and include data on the application of radioactive isotopes to research.

THE PROGRESS of science is characterized by the discovery and use of relatively few instruments or tools for application in research. Examples of such instruments are the analytical balance and the microscope which, when discovered, advanced science rapidly. Within the last fifteen years, however, two very useful instruments have been developed, the "cyclotron" and the "chain reacting pile." The cyclotron is capable of producing high-speed particles of matter of various descriptions and the pile of producing slow neutrons. The products of these instruments, radioactive isotopes, are new tools in the hands of the scientists. It has been stated that the range of usefulness of the products of the cyclotron and the pile is unsurpassed by any other tools at our disposal with the possible exception of the analytical balance and the microscope.

Pharmacy, as a profession, is chiefly the application of all branches of science to the production of medicinals and medicinal products, and since radioactive isotopes are applicable to all branches of science, isotopes have particular application in the various divisions of the pharmaceutical profession. In view of these facts, the pharmaceutical profession should be quick to awaken to the unique opportunities afforded by radioactive isotope "tracer" techniques.

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Isotopes have many important uses in the various fields of science such as development of power, atomic research, etc.; however, the pharmaceutical profession is primarily concerned with the use of products which are radioactive.

APPLICATIONS

Radioactive products have at the present time two very outstanding applications to pharmacy and pharmaceutical research: (1) as therapeutic agents when used properly and with necessary precautions and (2) as tracer elements. As therapeutic agents, radioactive isotopes by virtue of their radiations are, in the true sense of the word, a drug, and are so classified and regulated under Section 505 of the Federal Food, Drug, and Cosmetic Act.

The particles emitted when the isotope disintegrates are a source of penetrating radiation in the treatment of abnormal tissues, a circumstance in which the injury to unwanted tissue is notably greater than to normal essential tissue.

Radioactive Isotopes in Pharmaceutical Research.—In pharmaceutical research the use of radioactive isotopes in therapy offers several possibilities. Specific localizing compounds, which, by preferentially localizing in diseased tissue, will result in much greater radiation damage to the diseased than to normal tissue, need to be prepared and tested. A few such studies have been made, examples of which are iodine and certain gold colloids, but a great variety of compounds, and information concerning their concentration in various tissues, should be investigated. Perhaps much of this information may be forthcoming from data obtained from other studies.

A second possible application of radioactive isotopes concerning therapeutic agents is the production of modified forms of materials commonly prepared by manufacturing pharmacists. The effects of radiation in the production of modified strains of bacteria, viruses, fungi, and actinomyces, and such effects as the denaturation of proteins and inactivation of enzymes should be studied.

A third and very important study is that of the nature and method of treatment of radiation damage. In this direction drugs and necessary treatment should be developed to prevent or lessen radiation damage and sickness. This study is important for cases of possible human over exposure in radiation therapy, atomic energy work, and warfare.

Radioactive isotopes have already been used as diagnostic agents; however, much remains to be done toward extending this usage.

In regard to therapeutic usage of isotopes there is little direct commercial application in which the pharmacist, or manufacturing pharmacist will be interested since there is no immediate prospect of the use of isotopes in medicine in large amounts. The conditions in which isotopes have particular value therapeutically are rare at the present time and in such cases the dose is small and varied. The discovery of specific localizing agents as mentioned above might change this picture appreciably.

Application as Tracer "Elements".—Radioactive isotopes, as tracer "elements" rather than as therapeutic agents, have the principal applications to pharmacy and pharmaceutical research at the present time. A radioactive isotope of an element behaves identically with other isotopes of the element in all chemical and physiological processes. It labels without question the particular atoms one wishes to trace regardless of their incorporation into units with other atoms or into any combination in which one might desire to trace them. In other words, we have the ultimate in specificity for the tagging of an atom or element.

The advantages of the use of radioactive isotopes as tracer substances may be listed:

(1) The movement of tagged substances is easily followed using suitable detection devices.

(2) Small amounts of the tagged substance may be used. As an example, as little as 10^{-7} Gm. of iodine, 0.1 microgram, is sufficient for the tracer technique using radioactive iodine.

(3) It is often unnecessary to remove organs or tissues or to kill the organism in order to determine the presence and content of radioactive material.

(4) The detection of elements by their radioactivity is up to one million times as sensitive as by the usual methods of chemical or physical analysis. This means that roughly 10^{-18} to 10^{-20} Gm. of substance can often be readily detected.

Such sensitivity of measurement makes possible the intelligent study of problems which heretofore could not be solved by the use of existing methods or tools. In pharmacy and in pharmaceutical research in particular, we have many problems which lend themselves to the application of radioactive isotopes and which cannot be adequately solved by other existing methods.

Pure Pharmacy Research.—As a tool in pure pharmacy research, radioactive isotopes have widespread application. A few of the possible applications may be briefly mentioned here as illustrations of this point.

Researchers have long desired to know the rate of absorption of certain medicaments from various bases so that bases could be classified in this respect. Also it is desirable to know if certain medicaments pass through the normal skin and membranes at all and if so to what extent. Since such small amounts of materials are involved, isotopes offer about the only method of measuring such absorption. The rate of absorption of certain substances varies with the degree of irritation of the skin, thus the need for a method of measuring skin irritation is apparent.

The disintegration rate of tablets and enteric-coated pills and tablets *in vivo* may be most critically studied using radioactive isotopes. The problem of exactly when and where an enteric coat breaks down in the animal body is of considerable importance in determining the relative efficiencies of the various enteric coats.

The pharmaceutical profession has a variety of methods for the administration of a specific medicinal substance. The question of just which type of preparation gives the most satisfactory absorption or utilization of the medicinal can now be determined precisely by means of the tracer technique. These are but a few of the many examples which might be cited for the use of isotopes in pure pharmacy research.

Tracer Technique in Pharmacology.—In pharmacology as applied to pharmacy, the tracer tech-

nique offers even greater possibilities for study.

Wherever one desires to measure small amounts of substances this technique is particularly applicable. The determination of small amounts of residues on the skin and of the effect of various solutions upon the calcium-phosphorus depletion of teeth, both of which are to be reported on in subsequent papers, are excellent examples of tracer application. Studies such as these are next to impossible without the isotope tracer technique.

One of the greatest advantages which isotopes offer in pharmacology is making possible a study, not previously feasible, of the rate of absorption, excretion, alteration, breakdown, concentration in the blood, and per cent distribution in various organs of almost every synthetic medicinal substance. The same type of study may also soon be done with certain natural substances which are not readily synthesized, since a plant farm designed to grow plants in an atmosphere of radioactive CO_2 is now being set up in Chicago. This unit is being designed to produce natural drug principles containing radioactive atoms. To trace in the body such substances as vitamins, hormones, antitoxins, drugs, etc., which distribute themselves in tissue in minute quantities is impossible by ordinary analytical procedures. The value of such studies cannot be predicted; however, one can say that undoubtedly the contribution will be much greater than can be anticipated. Even the study of such a simple substance as ethyl alcohol would be a valuable contribution to pharmacy and medicine.

By use of the radioautographic technique more detailed tracer studies can be made, determining exactly in what cells within a particular organ the substance being studied concentrates.

Radioactive isotopes also lend themselves to toxicity studies, a major area of pharmacological study.

The study of the uptake, utilization, storage, mechanism of action, and need in the animal body of certain trace elements such as Cu, Co, Ni, Cr, etc. is now possible by the tracer technique. In short, we may say that radioactive isotopes are applicable to almost every pharmacological problem.

Practical Applications in Pharmaceutical Chemistry—In the field of pharmaceutical chemistry, the applications of radioactive isotopes are concerned primarily with the synthesis of substances which contain radioactive atoms which may then be traced. Such syntheses require special techniques and precautions, but are not considered difficult or dangerous.

The applications of isotopes in pharmaceutical chemistry, however, are not confined to synthesis. Radioactive isotopes offer the most sensitive quantitative method known and, as such, provide a number of practical applications. The determination of the solubilities of very slightly soluble compounds is not possible to the same degree by other existing methods. The use of isotopes for such determinations possesses the advantage that the presence of foreign ions in no way interferes with the measurements. The method may be applied without difficulty, for example, in determining the solubility of lead sulfate in the presence of calcium sulfate. The determination of the amount of a given substance that is absorbed or occluded during a precipitation or purification procedure offers considerable application. The determination of mere traces by ordinary chemical analysis, when it can be accomplished at all, requires tedious methods and is time consuming. Isotopes also lend themselves to a study of the behavior, chemical reactions, and properties of medicinal substances of unknown properties.

Colloids are extremely important in pharmaceutical and medicinal chemistry and radioactive isotopes are ideal for their study from almost every standpoint. Such studies would reveal considerable information about colloids which is not used or known today.

In conjunction with co-precipitation, elution, added carriers, and chromatographic absorption procedures, it will be possible to isolate, purify, and identify substances in small amounts from complex mixtures which heretofore was not possible. Such procedures used along with pharmacological studies will be invaluable to pharmaceutical chemical research.

Research Area in Pharmacognosy.—In the field of pharmacognosy, there are likewise many opportunities for applications of radioactive isotopes. Here again a few examples will suffice to illustrate the use.

Of prime importance at the present time is the production of natural plant products which are radioactive. With many alkaloids, glycosides, etc., this is only possible by the growth of plants from which these materials are obtained in an atmosphere of radioactive carbon dioxide or in a media containing the desirable tagging atom. The problems of production and isolation of such principles fall within the scope of the pharmacognosist. The use of radioactive isotopes in conjunction with the field of hydroponics offers a most fertile field for the production and study of natural plant medicinals. A study by the radioautographic technique of just what plant cells contain the active principles and at what periods the content is greatest in the various cells would be invaluable. The pharmacognosist can contribute to the production of radioactive antibiotics which in itself is an important research area. The rate of uptake, distribution, and elimination of various elements, radicals, and compounds in medicinal plants is a research project worthy of considerable study, especially in conjunction with the effect of various substances upon the active principles produced by the plant.

A study of the need of medicinal plants for certain trace elements and fertilizer needs likewise can now be investigated more thoroughly using the tracer technique.

Radioactive isotopes may also be applied to solve certain manufacturing problems, examples of which are the uniform distribution of drugs in various medicinal forms, mixing problems, and various quantitative problems.

SUMMARY

Briefly, we may say that radioactive isotopes are applicable to all branches of pharmaceutical research and by virtue of the very nature of pharmaceutical problems themselves are especially important.

It is hoped that the illustrations of the applications of radioactive tracers to pharmacy and

pharmaceutical research, as presented here, are of sufficient number and variety to show that this tool is capable of many diverse applications.

It is desirable that in the future a greater number of individuals interested in pharmaceutical research and the welfare of pharmacy make use of this excellent research tool and thereby facilitate the solution of many important pharmaceutical problems.

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The Use of a Microcrystalline Wax in Tablet Polishing. I.*

By W. CARL KELLEY†

Microcrystalline waxes have a multiplicity of uses. The production of a polish is very desirable on coated tablets. Two microcrystalline waxes from petroleum are produced by the Bareco Oil Company of Barnsdall, Okla. One melts at 170° F. and the other at 190° F. This paper deals with the wax of lower melting point and evidence is presented to show that when it is mixed with carnauba (or possibly some similar product) a satisfactory polish is produced.

THE POLISHING of coated tablets is an accepted practice in pharmaceutical manufacturing today. Polishing facilitates handling, makes the tablets more pleasing in appearance, and enhances the value of coating. Very little was found in the literature, however, pertaining to the processes or ingredients employed. A carnauba-beeswax mixture in a suitable solvent such as acetone was given by Bennett (1). Glyceryl tristearate was also mentioned as a polishing agent by Bennett. Stokes (2) recommended the application of a solution of carnauba wax in acetone, followed by a small amount of talc upon the evaporation of the solvent. A chloroform solution of carnauba wax and white wax has been suggested by Rose, in which about 100 grains of the waxes was used for fifty thousand five-grain tablets (3). A formula consisting of three parts of carnauba wax, two parts of beeswax, and two parts of paraffin was found in the textbook by Scoville and Powers (4).

Microcrystalline waxes from petroleum are produced in this country. They have been shown to be of value in the preparation of certain pharmaceutical preparations (5). It is the purpose of this paper to report on a preliminary study of the use of a microcrystalline wax for polishing tablets.

EXPERIMENTAL

A small improvised polishing drum, 10 inches in diameter and 8 inches deep, was lined on the inside with a light-weight canvas. It was rotated at the rate of twenty-two r.p.m., by an electric motor.

The microcrystalline wax used was "Bareco 170," which was furnished by the Bareco Oil Company, Barnsdall, Okla. (The 170 refers to the melting point of 170° F.) The Warren-Teed Pharmacal Company of Columbus, Ohio, supplied the brown-coated, unpolished tablets.

Bareco wax was melted, applied to the canvas inside the drum, and allowed to harden at 20°. Five hundred tablets were placed in the drum and the drum rotated. After a time, some modifications were made. Samples of twenty-five tablets each were taken at intervals and labeled as follows:

1. Polished for five minutes.
2. Polished for fifteen minutes.
3. Polished for twenty-five minutes
4. Polished for forty minutes.
5. Polished for one hour, added small amounts of powdered stearic acid and talc and allowed to rotate for an additional five minutes.
6. Same as (5) with an additional thirty minutes' rotation.

* Received May 22, 1947, from the College of Pharmacy, Ohio State University, Columbus, Ohio.
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7. Same as (6) but the tablets were placed in the drum lined with cheesecloth and rotated for forty-five minutes in an attempt to remove excess wax and improve the gloss.

The observations made during these tests showed that the tablets began to adhere to the waxed canvas and to each other after about twenty-five minutes of rotation. The wax did not produce a satisfactory gloss. Neither addition of the stearic acid and talc nor buffing with cheesecloth produced satisfactory results.

The formula was modified by melting Bareco wax and carnauba wax in equal proportion to replace the pure Bareco wax. Five hundred tablets were used as before. A good polish was produced after rotating them for approximately three hours.

The formula was again modified by using three parts of Bareco wax to one part of carnauba wax. The same procedure was followed as before. Rotating for from two to two and one-half hours gave very satisfactory results. The tablets had a polish that was comparable to the gloss found on tablets that are on the market.

A test was made to determine the possible effects upon the rate of disintegration when taken into the stomach. Distilled water was warmed to 37°. When the tablets were dropped into it, the color of the coating appeared in the water almost instantly

SUMMARY AND CONCLUSION

1. An excessive amount of wax was deposited on the tablets when the microcrystalline wax (Bareco wax 170) alone was used. This wax coating was dull in appearance and was also "tacky" or "sticky." By the method employed, the tablets used could not be polished satisfactorily by the microcrystalline wax only. Buffing the waxed tablet on cheesecloth does not greatly improve the gloss of the tablet and seems to be impractical.

2. Fifty per cent each of microcrystalline wax and carnauba wax was used to polish the tablet and the resulting gloss was satisfactory. The time required to polish the tablets seemed to be excessive.

3. A formula, consisting of 75 per cent microcrystalline wax and 25 per cent carnauba wax, produced a satisfactory polish on the tablets used. The polish did not interfere with disintegration of the tablets.

It is concluded, from this investigation, that Bareco 170° microcrystalline wax, in combination with carnauba wax, will produce a satisfactory polish on coated tablets.

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Book Reviews

Jubilee Volume Dedicated to Emil Christoph Barell. By the Scientific Workers of the Roche Companies. Basle, 1946. 468 pp. 15 x 23 cm.

This book is a typical European Jubilee volume dedicated, in this instance, to the president of F. Hoffmann-LaRoche and Company on the occasion of the 50th anniversary of his association with the firm. It represents reviews of scientific work done by members of the staff. Chemistry, pharmacology, mycology, bacteriology, and other scientific fields are represented and such variable compounds as vitamins, intestinal antiseptics, analgesics, adenine, antibiotics, and dicumarol are found in these papers. Eight of the papers are in German.

Inorganic Pharmaceutical Chemistry. By CHARLES H. ROGERS, TAITO O. SOINE, and CHARLES O. WILSON. Fourth edition. Lea and Febiger, Philadelphia, 1948. 704 pp. 23 illustrations. 15.5 x 24 cm. Price, $10.

The first edition of this well-known pharmaceutical text and reference book was published in 1930. Since that time it has undergone three revisions. The fact that it is now in its fourth edition should be a sufficient indication of its utility and value to pharmacists, teachers of pharmacy, and students in pharmacy. The fourth edition of this widely used textbook has been thoroughly revised and brought up to date. It not only reflects the most recent advances in inorganic pharmaceutical chemistry but conforms to the current editions of the United States Pharmacopœia and the National Formulary.

A significant feature of the new edition is the more detailed discussion of therapeutic value and medicinal uses of inorganic compounds and their preparations. The style and arrangement of the chapters are essentially the same as in previous editions. The book should, as it has in the past, serve a particularly useful purpose as a textbook for the correlation of general and inorganic chemistry with inorganic medicinal chemicals. The binding and printing are adequate and the book is well indexed. Those familiar with previous editions will not be disappointed when they examine and use the new fourth edition.

Scientific Edition

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Interaction of Autonomic Drugs on Isolated Mouse Intestine*

By LLOYD W. HAZLETON† and EMILY GODFREY†

The isolated mouse intestine as a tool for studying the actions of autonomic drugs was investigated in some detail. A comparatively simple method for studying the action of these drugs in the lumen, or with the lumen excluded, is described. In general, the mouse intestine is comparable to other isolated intestines in its reaction to these drugs. Of the drugs studied, only acetylcholine was totally inactive when confined to the lumen and none was destroyed in the lumen.

THE PHARMACOLOGY of autonomic drugs such as acetylcholine, atropine, and physostigmine was studied as early as 1906 and 1907 by Hunt and Taveau (1), Unger (2), and Magnus (3). The latter two investigated the action of the drugs on the mammalian intestines *in situ*, using the cat. Alvarez (4) in 1918 used five isolated segments of rabbit's intestines suspended in the same bath to show the varied reactions of seventy-five drugs on different parts of the intestine. Bernheim (5) in 1934 was among the first to study carefully the white mouse, using isolated small intestine.

Bernheim (5) studied the interaction of acetylcholine and adrenaline on the isolated small intestine and colon of practically all laboratory animals including mouse, rat, guinea pig, rabbit, fish, cat, and dog. Gunn (6) in his recent report on the antagonism of the two drugs used isolated rabbit intestine. To contrast the technique used by each of these workers is interesting because of the individual approach to the identical problem. Bernheim administered the drugs separately, which is the usual method, while Gunn mixed the drugs in varying concentrations, and both workers proved physiological antagonism between acetylcholine and adrenaline. A preliminary report by Hazleton and Godfrey (7) described a method for studying the action of autonomic drugs placed in the lumen of mouse intestine and presented some of the more fundamental results. Somewhat later Meyer and McEwen (8) used a modified Trendlenburg method to study the action of drugs on the serosa and mucosa of isolated rabbit and guinea-pig intestine in an attempt to differentiate the finer responses of the intestinal muscles.

The objective of the following report is to present the results of additional studies employing the mouse method and to describe some of the characteristic interactions of the various autonomic drugs.

* Received Feb. 24, 1948, from the Kalusowski Memorial Research Laboratory, School of Pharmacy, The George Washington University, Washington, D. C. Acknowledgment is made of a grant from the Proprietary Association in support of this study.

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APPARATUS AND TECHNIQUE

Tubular segments of small intestine were suspended between a fixed and a freely moving hollow glass cannula as illustrated in Fig. 1. These were placed in a smooth-muscle chamber contained in a constant temperature tank at 37.5°. Fluid or air could be placed in the lumen by means of a tuberculin syringe joined to the top of the fixed cannula by a short rubber tube. The cannulae used in these experiments required a volume of 0.4 cc. to fill them and the intestinal segment to a level approximating the fluid level of the chamber. This volume was used consistently for placing drugs into the lumen. The open end of the moving cannula extended sufficiently above the fluid level of the chamber to permit maximum contraction of the muscle without immersing the cannula. Since the upper end of each cannula was open to the air, the fluid pressure in the lumen was at equilibrium with the external pressure. The chamber contained oxygenated Locke-Ringer's solution while the drugs were dissolved in distilled water. When drugs were placed in the lumen there thus existed an osmotic gradient favoring interchange between the lumen and the chamber. Freedom of experimental design

TABLE I.—EXPLORATORY TESTS TO DETERMINE THE EFFECTIVE DOSAGE RANGE AND INTERACTION OF ONE OR MORE DRUGS WHEN PLACED IN THE BATH[a]

Drug	Conc.	Vol./ 100 Cc.	Followed by	Conc.	Vol./ 100 Cc.	Results
Acetylcholine	1:10,000	1.0	.....	....	...	Maximum typical contraction
	1:10,000	0.02	.....	....	...	Standard dose giving uniform contraction[b]
	1:10,000	0.01	.....	.....	...	Minimum contraction
	1:10,000	0.02	Atropine	1:1,000	{0.01 0.02	Relaxation
	1:10,000	0.05	Epinephrine	1:1,000	0.05	Drop in base line and cessation of activity for a short time
Atropine	1:1,000	0.01	.....	....	...	Relaxation with level base line
	1:10,000	0.01	.....	....	...	Relaxation with level base line
	1:100,000	0.01	.....	....	...	Relaxation with level base line
	1:1,000	0.01	Acetylcholine	1:10,000	0.01	No acetylcholine contraction
	1:1,000	0.01	Acetylcholine	1:10,000	0.02	After repeated washings, normal response
	1:1,000	0.02	Acetylcholine	1:10,000	1.0	Acetylcholine response equal to that of standard dose
Physostigmine	1:1,000	0.1	.....	....	...	Paralysis
	1:10,000	0.1	.....	....	...	Paralysis
	1:100,000	0.1	.....	....	...	Paralysis
	1:1,000,000	0.1	.....	....	...	Paralysis
	1:10,000,000	0.1	.....	....	...	Enhancement of activity
	1:10,000,000	0.1	Acetylcholine	1:10,000	0.005	Response comparable to the minimum typical contraction of acetylcholine
Prostigmine	1:4,000	0.1	.....	....	...	Paralysis
	1:8,000	0.1	.....	....	...	Paralysis
After several washings of bath			.....	....	...	Enhancement and prolongation of acetylcholine activity
	1:4,000	0.1	Acetylcholine	1:10,000	0.01	More uniform and prolonged reaction than physostigmine. Contraction comparable to 0.02 of acetylcholine
Epinephrine	1:1,000	0.01	.....	....	...	Drop in base line, temporary decrease of activity
	1:10,000	0.01	.....	....	...	Drop in base line, temporary decrease of activity
	1:100,000	0.01	.....	....	...	Drop in base line, temporary decrease of activity
	1:10,000	0.01	Acetylcholine	1:10,000	0.05	Response comparable to standard dose of acetylcholine when epinephrine was not present

[a] Except as noted, the bath was not washed prior to addition of the second drug.
[b] The standard dose designates that dose which produced a fairly uniform but submaximal response in satisfactory muscles.

was somewhat limited by the fact that the only means of removing drugs from the lumen was to expel them into the chamber, with air or fluid from the syringe.

Segments of ileum 3 to 4 cm. in length were obtained immediately after sacrificing mice of either sex by cervical dislocation. The segments were freed of mesentery and fecal material, suspended as described above, filled with Locke-Ringer's solution and allowed to relax for the normal tracing. Muscles which did not exhibit some degree of spontaneous activity were rejected as unsatisfactory.

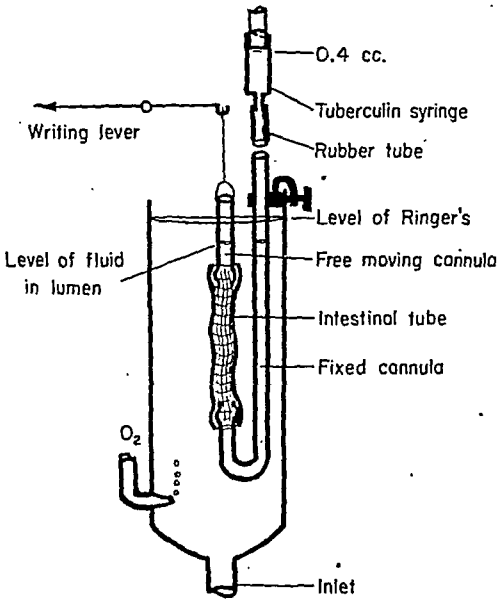

Fig. 1.—Diagram of apparatus for studying the effect of drugs in the lumen of the isolated mouse intestine. In practice two muscles were placed in each bath, one serving as a control.

To avoid thermal shock all solutions were maintained at 37.5° by immersing the containers in the constant temperature tank. Under these conditions the segments remained active over periods of several hours.

Two separate muscles were usually suspended in each chamber, the first being the test segment and the second, the control. The control provided a simultaneous normal response for comparison with the treated muscle response under the various conditions of the experiment. In addition it served to detect the inadvertent contamination of the chamber by the drugs.

RESULTS

The results to be presented here are largely confined to the methodology and the more obvious action mechanisms. To avoid excessive discussion these will be presented by a series of figures and tables with individual consideration of the illustrative points.

Table I presents the results of exploratory tests to determine the effective doses and interactions of drugs placed in the chamber. As might be expected, these results correspond well with those obtained in the usual smooth-muscle experiments in other animals where the exterior and lumen both contact the solution. No evidence was obtained that exclusion of the lumen significantly altered the results. In all but extremely small doses the muscles were paralyzed by physostigmine and prostigmine, and it was found that best enhancement of acetylcholine activity was obtained after these drugs had been removed from the chamber. The usual atropine-acetylcholine antagonism was observed, but it is interesting to note that following removal of atropine from the chamber the standard dose of acetylcholine was ineffective while (*a*) large doses of acetylcholine overcame the residual atropine effect and (*b*) repeated washings restored the effectiveness of the standard dose.

Table II combines several phases of the problem by selecting the more classical actions and interac-

TABLE II.—ACTION AND INTERACTION OF AUTONOMIC DRUGS UNDER VARIOUS CONDITIONS

Action of Drug in Lumen of Test Muscle, Volume—0.4 Cc.				Interaction of Acetylcholine (1:10,000 conc.) When Placed in the Bath While the First Drug is Present in the Lumen of the Test Muscle			After Washing the Bath of All Acetylcholine, Action of Both Muscles on Expelling the Drug from Lumen of the Test Muscle	
Drug	Conc.	Test Muscle	Control Muscle	Quantity of Acetylcholine, Cc.	Test Muscle	Control Muscle	Test Muscle	Control Muscle
Acetylcholine	1:10,000	N	N	....	..	.	+	+
Atropine	1:1,000	—	N	0.01–1.0	—	+	—	—
	1:10,000	—	N	0.01–1.0	—	+	—	—
	1:100,000	—	N	0.01	N	+	—	—
	1:100,000	—	N	1.0	+	+	—	—
Physostigmine	1:1,000	—	N	0.01–1.0	—	+	—	—
	1:10,000	—	N	0.01–1.0	—	+	—	—
	1:100,000	—	N	01–1.0	—	+	N	N
	1:1,000,000	N	N	0.005	+	+	N	N
	1:10,000,000	N	N	0.005	+	+	N	N
Prostigmine	1:4,000	—	N	0.005–1.0	—	+	N	N
	1:8,000	—	N	0.005	+	+	N	N
Epinephrine	1:1,000	—	N	0.02	+	+	—	—
	1:10,000	—	N	0.02	+	+	—	—
	1:100,000	N	N	0.02	+	+	—	—

N—normal activity.
+—contraction typical of drug involved.
——relaxation and/or cessation of normal activity which was of varying periods of duration.

tions of autonomic drugs. Several of these and a few which do not lend themselves to the tabulation deserve consideration.

The first portion of the table summarizes activity of drugs confined to the lumen of one intestinal tube and indicates that there is no interchange of drugs between the lumen and the surrounding bath since in no instance was the control muscle affected.

The center portion of the table is concerned with the interaction between lumen-contained drugs and acetylcholine. In general, this confirms the non-interchange of drugs since the control muscle responds to acetylcholine within normal limits. In the test muscle a typical atropine-acetylcholine antagonism is obtained throughout the normal range of doses, as illustrated in Fig. 2. By varying the ratio of doses, however, a gradation of antagonism is obtained. Physostigmine and prostigmine exhibit antagonistic and synergistic properties comparable to those obtained with these drugs in the bat . It was also observed that in addition to sensitizing the action, the prolongation of acetylcholine response was marked following the smaller (nonparalytic) doses of these drugs.

The interaction between lumen-contained epinephrine and acetylcholine is perhaps the most interesting of the series. Even very active muscle strips were completely relaxed by epinephrine in the lumen but responded in normal manner following the addition of acetylcholine to the chamber. This is illustrated in Fig. 3. The ability of epinephrine to produce complete relaxation without material interference with the acetylcholine reaction is in sharp contrast to the action of atropine under similar circumstances. In the very active muscles atropine reduces spontaneous activity but in so doing completely blocks the normal acetylcholine response. It was further observed that following the explusion of epinephrine (1 to 1000) from the lumen and washing the epinephrine from the bath the administration of acetylcholine into the lumen produced a very strong contraction. Although the explanation for this phenomenon is not readily apparent, it is the only circumstance under which an acetylcholine response was elicited by the drug in the lumen.

The third portion of Table II summarizes the responses obtained following expulsion of the lumen contained drugs into the bath. The actions obtained under these conditions, particularly those in the control muscle, indicate that none of the drugs studied was inactivated by confinement within the lumen (Fig. 4). Under these conditions the most dilute solutions of physostigmine produce an increase in activity which is not antagonized by normal doses of atropine. It is to be assumed that this is due to the continued activity of residual drug in the lumen of such intensity that externally applied atropine is incapable of complete antagonism.

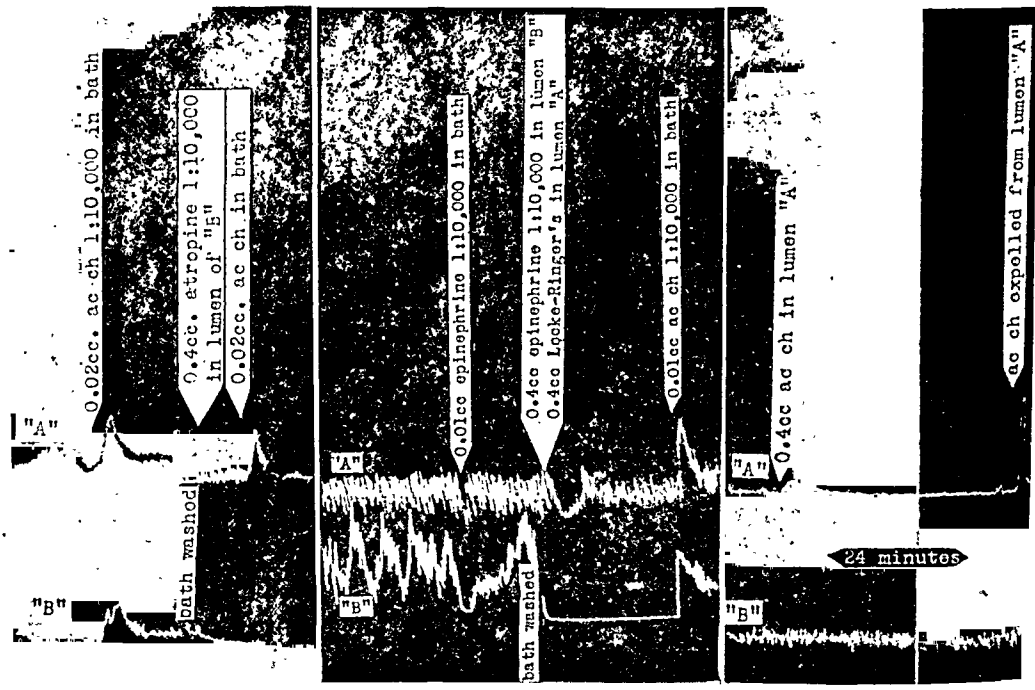

Fig. 2 Fig. 3 Fig. 4

Fig. 2.—Showing action of atropine in the lumen, noninterchange between lumen and bath, and antagonism of acetylcholine in bath.

Fig. 3.—Showing action of small dose of epinephrine in the bath, prolonged relaxation from epinephrine in the lumen, and absence of antagonism to small dose of acetylcholine in the bath.

Fig. 4.—Demonstrating the ineffectiveness and stability of acetylcholine in the lumen, and the function of a control muscle.

SUMMARY

In general, the response of the mouse small intestine to various classical autonomic drugs may be considered to be analogous to those of other mammals. The convenience, cost, and availability of this investigatory tool would seem to merit more widespread usage than it has enjoyed in the past.

By use of a simplified technique for the study of action of drugs in the lumen it appears that several interesting fields of more detailed investigation have been revealed. The method is such that it eliminates many of the complicated features of those previously used for this approach to the problem, and while it is subject to further improvement it appears to be reliable for at least fundamental observations. Not the least of the desirable features is the use of a continuous control muscle, making possible the avoidance of inadvertent errors and the evaluation of the various responses under controlled conditions. It is to be hoped that this report will stimulate interest both in the mouse intestine as a tool and in the method which has been applied.

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Spectrophotometric Determination of Procaine in Procaine Penicillin G*

By C. V. ST. JOHN†

In a search for a rapid method for the determination of procaine in procaine penicillin G a spectrophotometric method was developed. A series of lots analyzed by the spectrophotometric method and by the chloroform extraction-titrimetric method shows comparable results. Measurements were made on a Beckman spectrophotometer at 290 mμ.

DURING the experimental stages of the development of procaine penicillin (3) it was necessary to determine the procaine content of the final procaine penicillin G to determine whether or not excess procaine had been carried along with the precipitate. Since additional procaine greatly increases the toxicity of the product this determination was of great importance.

The conventional method (2) for the determination of procaine involves the addition of an excess of ammonium hydroxide, extraction of the procaine base with chloroform, evaporation of the chloroform, addition of a known amount of hydrochloric acid, and back titration of the excess with sodium hydroxide. Since this involves much time a spectrophotometric study of the absorption spectra of procaine was made. It was found that the peak of the absorption band for procaine exists at 290 mμ.

EXPERIMENTAL

Apparatus and Solution.—Transmittancy measurements were made with a Beckman quartz spectrophotometer (1) equipped with 1.000-cm. quartz cells and an ultraviolet radiation source operating with a spectral band width of approximately 1–5 mμ. Distilled water was used in the reference cell.

A standard solution of procaine hydrochloride was made from a lot of material that analyzed 100.0% by the chloroform shake-out procedure. This solution was freshly prepared whenever used.

Effect of Interferences.—A certain amount of interference is to be expected in any method of this

* Received May 7, 1948, from Antibiotics Manufacturing and Development Dept., Eli Lilly and Company, Indianapolis, Ind.

† The author is indebted to D. E. Flick for analyzing the series of lots of procaine penicillin by chloroform extraction-titrimetric method.

type. It is understood that any compounds that absorb ultraviolet radiation at 290 mμ will interfere. Penicillin G does not interfere at the concentration equivalent to the amount of procaine being measured. Acid decomposition of the equivalent amount of penicillin G gives some interference as shown in Fig. 1; however, in this laboratory no interference has been encountered in the final product. If interference exists some method of removing or correcting for this interference must be developed

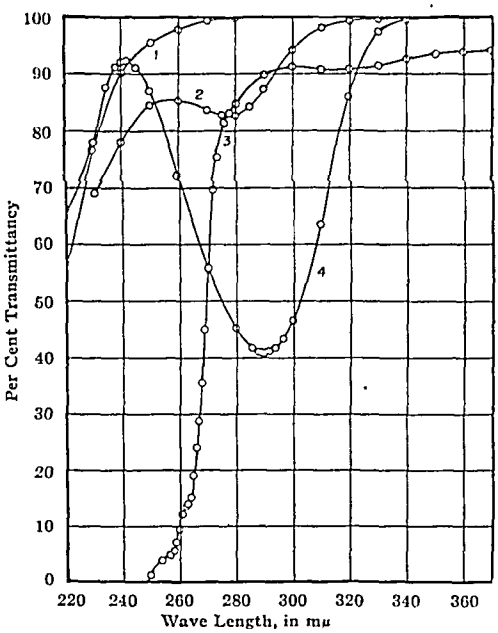

Fig. 1

1. Approximately 0.008 mg. potassium penicillin per ml.
2. Approximately 0.008 mg. potassium penicillin per ml. decomposed with HCl and heat.
3. Approximately 2.0 mg. potassium penicillin per ml.
4. 0.005 mg. procaine base per ml.

Conformity to Beer's Law.—The system conforms to Beer's law at concentrations within the range of the instrument.

Effect of *p*H.—The *p*H range of the crystalline product (5.0–7.5) as specified by the Federal Food and Drug Administration does not significantly change the *p*H of the final dilution. It is interesting to note that on all the material produced in our laboratories the *p*H of the crystalline product has been between 5.7 and 6.1.

Effect of Concentration.—The lower limit of procaine concentration which can be determined by this method is fixed by the maximum cell length and interferences; the upper limit is fixed by the error encountered by repeated dilution. The concentration range of the final dilution for a 1-cm. cell is 0.001–0.010 mg. of procaine base per ml.

Comparison of Spectrophotometric and Chloroform Extraction-Titrimetric Method.—A series of final lots of procaine penicillin was analyzed by both methods by separate operators. The results ar shown in Table I. The results obtained show ver little deviation; however, care must be taken t clean the cells frequently with dichromate-sulfuri acid cleaning solution and to make careful adjust ment of the instrument. Cell calibration shoul also be checked. The arithmetical average of th readings for several adjustments of the instrumen on each of two fillings of the cell should be taken If care is exercised results comparable to those show in Table I can be expected.

TABLE I.—COMPARISON OF SPECTROPHOTOMETRI METHOD WITH CHLOROFORM EXTRACTION-TITRI METRIC METHOD

Lot No.	Chloroform Extraction Method, % Procaine	Spectrophotometric Method, % Procaine	Deviation, %
1	40.0	39.9	−0.25
2	39.7	39.7	0.00
3	39.8	39.9	+0.25
4	39.8	39.9	+0.25
5	40.3	40.2	−0.25
6	40.2	39.9	−0.75
7	39.7	40.0	+0.75
8	39.8	40.3	+1.25
9	39.8	40.2	+1.00
10	40.4	40.3	−0.25
		Average Deviation	±0.5

Recommended Procedure

Weigh accurately approximately 50 mg. of procaine penicillin G and transfer to a 100-ml. volumetric flask. Dilute to volume with distilled water and shake until completely dissolved. Transfer 5 ml. of this dilution to a 250-ml. volumetric flask, dilute to volume, and measure the optical density of the resulting solution at 290 mμ against water in the reference cell. Obtain the concentration of the solution by calculation from an extinction coefficient determined on a sample of pure procaine hydrochloride or a sample of pure procaine penicillin G analyzed by the chloroform shake-out titrimetric procedure.

SUMMARY

A spectrophotometric method for the determination of procaine in procaine penicillin G has been described. The accuracy of the method compares very favorably with the conventional method, especially when care is exercised in dilution, cleaning of absorption cells, and in the adjustment of the instrument. Interference has not been encountered in the analysis of the final product. The procedure is simple and requires less time than the conventional method.

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Pharmaceutical Applications of Isopropyl Alcohol. III. Solubilities of Sedatives, Hypnotics, and Sulfonamides*

By HENRY M. BURLAGE†

In a previous paper (1) the solubilities of local anesthetics in isopropyl alcohol have been reported. The present work is a continuation of the determinations of this constant and is applied to official and nonofficial sedatives, hypnotics and sulfonamides. The procedure employed is outlined therein (1). The results are shown in Tables I and II.

TABLE I.—SOLUBILITIES OF SEDATIVES AND HYPNOTICS IN ISOPROPYL ALCOHOL

Name of Compound	Manufacturer	Solubility, Gm./100 Cc. of Solution
Barbituric Acid Derivatives		
Alurate N. N. R.	Hoffman-LaRoche	30.5430
Sodium Alurate N. N. R.	Hoffman-LaRoche	25.5750 (d.)
Sodium Amytal	Lilly	32.1730
Barbital U. S. P.	Merck	11.5772
Veronal N. N. R.	Winthrop	11.2520
Barbital Sodium	Merck	0.3008
Veronal-Sodium N. N. R.	Winthrop	0.3710
Dial N. N. R.	Ciba	8.2990
Ipral Calcium N. N. R. (Probarbital)	Squibb	0.2920
Ipral Sodium N. N. R.	Squibb	0.8750
Neonal N. N. R.	Abbott	53.0670
Ortal Sodium N. N. R.	Parke Davis	27.9500
Pentobarbital Sodium	Gane's	2.5010
Pentothal Sodium N. N. R.	Abbott	19.8370 (d.)
Phenobarbital U. S. P. (Phenobarbitone)	Gane and Ingram	11.7012
	Merck	11.6128
Luminal N. N. R.	Winthrop	11.3480
Phenobarbital Sodium	Gane and Ingram	14.7910
Luminal Sodium N. N. R.	Winthrop	14.5950
Sandoptal N. N. R.	Sandoz	30.2900
Seconal Sodium N. N. R.	Lilly	60.7620
Vinbarbital Sodium N. N. R.		
Delvinal Sodium	Sharp and Dohme	9.4900 (d.)
Compounds Containing Bromine		
Bromural N. N. R.	Bilhuber Knoll	9.7560
Carbromal N. F.	Merck	7.1620 (d.)
Adalin	Winthrop	6.4360
Hydantoin Derivatives		
Diphenylhydantoin Sodium U. S. P.		
Dilantin Sodium	Parke Davis	4.8810
Sulfonmethanes		
Sulfonethylmethane	Merck	11.2170
Sulfonmethane N. F.	Merck	2.1290

(d.) denotes decomposition.

TABLE II.—SOLUBILITIES OF SULFONAMIDES IN ISOPROPYL ALCOHOL

Name of Compound	Manufacturer	Solubility, Gm./100 Cc. of Solution
Sulfonamide Compounds		
Sulfadiazine U. S. P.	Squibb	0.0430
Sulfaguanidine U. S. P.	Squibb	0.1770
Sulfamerazine U. S. P.	Sharp and Dohme	0.1740
Sulfanilamide U. S. P.	Gane and Ingram	0.7970
Sulfapyrazine N. N. R.	Mead Johnson	0.0290
Sulfapyridine U. S. P.	Merck	0.1750
Sulfathiazole U. S. P.	Merck	0.5750
Succinylsulfathiazole U. S. P.		
Sulfasuxidine N. N. R.	Sharp and Dohme	0.5690
Sulfamyd	Schering Corp.	5.6650
Sulfonamide Sodium Salts		
Sulfadiazine Sodium U. S. P.	Squibb	0.1860
Sodium Sulfamyd	Schering Corp.	0.6290
Sulfapyrazine Sodium N. N. R.	Mead Johnson	0.7080

SUMMARY

The solubilities, in isopropyl alcohol, of 28 sedatives and hypnotics and of nine sulfonamide compounds and three sulfonamide sodium salts, on the American market, are reported on the basis of weight in volume.

REFERENCE

(1) THIS JOURNAL, 36, 16(1947).

* Acknowledgment is made to Enjay Company, Inc., of New York for funds to carry on this study and to the various manufacturers who generously furnished the supplies of the sedatives and hypnotics used.

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Effect of Pruning and Exfloration on the Growth and Alkaloidal Content of *Datura metel* Linn.*

By S. PRASAD†

Pruning and exfloration are known to affect vegetative and reproductive vigor in plants. While much work has been carried out during recent years on a variety of economic plants, no such study has been made of medicinal plants. The present investigation deals with the effect of pruning and exfloration on the growth and alkaloid formation in *Datura metel.*

PRUNING and exfloration are known to affect the vegetative and reproductive vigor in plants. A good deal of work has been carried out during recent years on a variety of economic plants (1–10). Severe pruning, in general, produced inhibitory effects and reduced the yield; mild operation, on the contrary, increased the yield. Removal of flower stalks and axillary buds, in case of tobacco, resulted in an increase of the size of the leaves (13), and in tomatoes defruiting or exfloration greatly increased the size of the treated plants (5, 6). In soy beans, Austin (11) noted no such stimulating effect.

Barring crop plants and fruit trees, little attention has so far been paid to investigating the effects of pruning and exfloration on medicinal plants. While studying the effect of mineral deficiency on *Datura alba*[1] (14), it was discovered that maximum alkaloid content was formed at the time of flowering and thereafter the alkaloidal content decreased. It was, therefore, decided to ascertain if the yield of the leaves and the alkaloidal content could be increased by pruning and/or suppressing the flower bud formation. Observations recorded in these directions are presented in this contribution

EXPERIMENTAL

Healthy seeds of *Datura metel* were selected with respect to their uniformity in size and weight and germinated in well-prepared seed beds. A month later when seedlings were of 3–4 cm. in height, they were transplanted in test plots 15 × 18 ft. in size; 42 plants were sown in each plot at a distance of $2^3/_4$ ft. each way. Treatments, seven in number, were replicated four times. Each block thus contained seven randomized plots, receiving the following treatments:

1. Control (no treatment given).
2. Most of the branches pruned (heavy pruning).
3. Only one branch from the base pruned (mild pruning).
4. Flower buds removed from only one branch, *viz.*, the main half stem from the base.
5. Flower buds removed from the whole plant (early exfloration).
6. Flower buds removed from the whole plant at a later stage (late exfloration).
7. Flower buds removed from the whole plant early and also treated a month before exfloration with ammonium sulfate (20.6% N) at the rate of 600 Gm. per plot.

After one and a half months when the seedlings were established fully in the plots and the plants bore a few branches, pruning was done. Care was taken that no injury to the main stem was caused. Exfloration was done on some plants at an early stage and on others at later stages when the flower buds were formed. All effects were compared against the control which consisted of unpruned and unexflorated plants.

The procedure adopted for measuring plant characters and for estimating alkaloids was the same as employed before (14) for this plant species.

RESULTS

General Growth Characters.—The control plants showed their normal growth and development and formed flowers when they were four months old; later at intervals of twelve to fourteen days new crops of flowers were formed which bore fruits. Plants from which most of the branches were removed (treatment no. 2) were severely injured and were not able to produce new shoots until fairly late in the season; it was about a month and a half after pruning that new shoots developed on the main stem, more commonly at the base of the pruned branches. Treatment No. 3, where only one branch from the base was removed, showed profuse growth and compared favorably with the control. Pruning failed to increase the growth of these plants; flowering was also delayed in these pruned plants.

Delayed flowering was a distinct feature in all exflorated plants. In treatment no. 5, where flower buds were continuously removed, new flower buds developed a week later than those of the normal plants. Even under treatment no. 4, where only one branch from the base was exflorated, delayed flowering was noted by four to five days; while nonexflorated branches on such plants exhibited intense blossoming, the exflorated ones on the same plant showed only the presence of buds at a well-developed stage. In plants where flower buds were removed late (treatment no. 6), flowering was delayed by two to three days. In treatment no. 7, where early exfloration was preceded by treatment

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[1] According to H. A. Timmerman, *Datura alba* Nees is *Datura metel* Linn. (*Pharm. J.*, 1927, pp. 571–574). Hence to maintain the priority, *D. metel* Linn. has been used in this contribution, for *D. alba* Nees, the latter name having been employed by the writer in his previous publications.

with nitrogenous fertilizer, production of flowers was delayed by about a week.

Vegetative growth was very much increased in all the exflorated plants. Height and thickness of stem and number and size of the leaves and the branches were increased over those of the normal plants.

Stem Elongation.—Height in severely pruned plants (treatment no. 2) was significantly decreased at all stages in the life cycle, in response to heavy pruning (Table I). In mildly pruned plants, pruning affected height adversely in the beginning; at later stages, however, height approached more or less the control. In exflorated plants, height of plants was significantly increased at the majority of the stages. Greatest increase in stem elongation was noted in exflorated plants treated with nitrogen (treatment no. 7). Treatment nos. 5, 4, and 6 followed next in order.

Leaf Number.—Number of leaves was significantly reduced in pruned plants. When pruned mildly, reduction was not significant due to formation of new branches and leaves. In exflorated plants, number of leaves increased at all stages; greatest increase was noted in early exflorated and nitrogen-treated plants. Next in order of increase were the plants of treatment nos. 5, 6, and 4.

Dry Weight of Plants.—Dry matter of entire plant of treated and control sets was likewise affected in the order mentioned above. Heavily pruned plants (treatment no. 2) were greatly affected; dry weight production was significantly reduced at practically all the stages of the life cycle. Mildly pruned plants

TABLE I.—AVERAGE PLANT CHARACTERS OF *DATURA METEL*, AS AFFECTED BY EXFLORATION AND PRUNING

	Treatment Numbers							
	1	2	3	4	5	6	7	
Age, Mo.	Control	Heavy Pruning	Mild Pruning	Exfloration in Half Stem	Early Exfloration	Late Exfloration	Early Exfloration and Treated with Ammonium Sulfate	S. E.[c]
				Stem Height in Inches				
2	4.85	.....	.....	.....	.....	.....	.....	.....
3	8.60	7.40	7.85	.....	.....	.....	.....	.....
4	19.85	10.55[a]	16.20[a]	22.25[b]	23.60[a]	20.10	25.05[a]	±1.024
5	26.75	16.25[a]	25.25	34.08[b]	41.13[a]	30.13	46.50[a]	±2.817
6	42.20	29.10[b]	40.85	52.60[b]	58.60[a]	52.80[b]	60.20[a]	±5.220
				Leaf Number				
2	12.5	.....	.....	.....	.....	.....	.....	.....
3	25.5	6.0	10.8	.....	.....	.....	.....	.....
4	45.0	10.5[a]	28.5[a]	50.0[a]	55.5[a]	45.00	62.00[a]	±0.850
5	85.4	20.0[a]	20.8[a]	110.0[a]	128.0[a]	104.35[a]	140.5[a]	±4.247
6	98.8	35.0[a]	68.4	220.0[a]	250.5[a]	238.50[a]	300.0[a]	±16.983
				Dry Weight of Entire Plant in Gm.				
2	1.399	.....	.....	.....	.....	.....	.....	.....
3	16.072	5.389	9.076	.....	.....	.....	.....	.....
4	39.525	10.215[a]	35.953	44.363[b]	54.250[a]	42.159	49.491[a]	±2.256
5	80.203	32.165[a]	76.593	90.297[a]	113.508[a]	94.290[a]	115.535[a]	±3.385
6	162.164	49.240[a]	152.300	189.680[a]	241.259[a]	226.258[a]	288.540[a]	±7.803
				Dry Weight of Leaf in Gm.				
2	1.104	.....	.....	.....	.....	.....	.....	.....
3	9.452	2.074	4.568	.....	.....	.....	.....	.....
4	22.280	3.240[a]	20.465	24.505	30.056[a]	23.105	35.520[a]	±2.048
5	35.700	10.603[a]	39.375	40.732[b]	50.825[a]	41.035[b]	53.075[a]	±2.429
6	68.873	20.450[a]	63.165	83.810[a]	103.103[a]	96.823[a]	124.568[a]	±3.102
				Dry Weight of Stem in Gm.				
2	0.210	.....	.....	.....	.....	.....	.....	.....
3	5.215	2.105	3.158	.....	.....	.....	.....	.....
4	14.150	4.250[a]	11.580	16.500	20.348[a]	15.804	23.846[a]	±1.462
5	36.868	17.095[a]	29.773[a]	41.470[b]	52.543[a]	41.978[b]	52.973[a]	±1.820
6	72.728	23.073[a]	65.533[a]	84.998[a]	108.120[a]	102.943[a]	133.130[a]	±2.221
				Dry Weight of Root in Gm.				
2	0.085	.....	.....	.....	.....	.....	.....	.....
3	1.405	1.210	1.350	.....	.....	.....	.....	.....
4	3.105	2.725	2.908	3.358	3.846	3.250	4.125[b]	±0.423
5	7.635	4.668[a]	7.445	8.095	10.140[b]	11.278[a]	9.848[b]	±0.857
6	20.563	5.718[a]	23.603	20.870	30.018[a]	26.593[b]	30.843[a]	±3.012
				Percentage Alkaloidal Content				
4½	0.2250	0.2045	0.2168	0.3225[a]	0.3856[a]	0.3425[a]	0.4025[a]	±0.0180
5½	0.2026	0.1920	0.1956	0.3410[a]	0.3824[a]	0.3500[a]	0.3850[a]	±0.0195

[a] Difference when compared with the control significant at 1%.
[b] Difference when compared with the control significant at 5%.
[c] Standard error of difference of means.

were affected only in the beginning (fourth month). Exflorated plants significantly increased in dry weight at all stages. Treatment no. 6, however, where exfloration was performed later, failed to increase significantly dry matter in the fourth month. Greatest dry matter production took place in treatment no. 7; treatment nos. 5, 6, and 4 followed in order.

Dry weight of component organs was affected in more or less the same order. Heavily pruned plants (treatment no. 2) showed significant reduction in leaf and stem weight at all stages (Table I); mildy pruned ones (treatment no. 3) exhibited significant reduction in stem weight only at fifth and sixth months and very little effect in their leaf weight. Increase in dry weight of exflorated plants was significant in case of treatment nos. 5 and 7 at all stages. In nos. 4 and 6, where partial or later exflorations were tried, significant effect was observed only at later periods, namely the fifth and sixth months of their life cycle. On dry weight of roots, partial exfloration or mild pruning had practically no effect; severe pruning, however, lowered dry matter and exfloration increased it significantly.

Alkaloidal Content.—Pruning of *Datura metel* did not affect the alkaloidal content of the leaves. Exfloration, early or late, on the other hand, brought about an increase (Table I.) This is in keeping with the fact that at the time of flowering, the alkaloidal content is maximum, but with the formation of the fruits and seeds a gradual decrease takes place, apparently due to the translocation of alkaloids from leaves to flowers and thence to fruits and seeds (Cf. Farr and Wright, 1904; Mueller, 1914). Exfloration checks such translocation; the alkaloidal content of the leaves, in consequence, remains at a high level throughout.

TABLE II.—THE EFFECT OF EXFLORATION IN TREATMENT NUMBER 4 (HALF EXFLORATED PLANTS)

Plant Characters[a]	Fourth Month Exflorated	Fourth Month Nonexflorated	S. E.	Fifth Month Exflorated	Fifth Month Nonexflorated	S. E.	Sixth Month Exflorated	Sixth Month Nonexflorated	S. E.[d]
Shoot length, in.	16.40	14.85	±1.050	27.55[b]	22.20	±2.055	45.50[b]	38.50	±2.500
Leaf No.	29.50[c]	20.50	±1 550	69.09[c]	50.80	±3.500	130.50[c]	89.50	±5.250
Dry weight of stem, Gm.	8.450[b]	6.058	±1.005	20.350[b]	16.450	±1.450	38.256[c]	31.409	±2.056
Dry weight of leaf, Gm.	13.203[c]	11 302	±0.596	22.527[c]	18.205	±1.245	45.700[c]	38.010	±2.120
Alkaloidal content, %	0.3305	0.3205	±0.0058	0.3450	0.3360	±0 0060			

[a] Only mean values are given. The length and dry weight of the basal part of the stem common to the exflorated and nonexflorated branch have been excluded.
[b] Significant at 5%.
[c] Significant at 1%.
[d] S. E. refers to the standard error of mean.

DISCUSSION

From the foregoing it appears that pruning of *Datura metel* has no beneficial effect. On the contrary it impairs the vigor of the whole plant to such an extent that the plant is unable to compensate even at later stages. Mild pruning, however, stimulates growth, but does not lead to any increase over the control plants. Exfloration, on the other hand, brings about an all-round beneficial effect on the whole plant. Stimulating effects of exfloration on the vegetative growth and alkaloidal content are marked even in partially exflorated plants—exflorated branch significantly increases in height, leaf number and dry weight of stem and leaf as compared to the nonexflorated branch of the same plant (Table II). On alkaloid content also, exfloration tends to bring about significant increases.

Results obtained by other investigators (12, 6–10), point out that structural features of the plants in part also determine the relationship between limits of vegetative and reproductive growth. Thus in plants of determinate habit, the inflorescence utilizes all the apical meristem and hence arrests elongation. In plants of indeterminate growth habit, on the other hand, stem elongation continues until the developing fruits begin to utilize the available food supply. In the latter case again, wider differences are noted from species to species. Thus where fruiting takes place once for all in the life cycle, as in crop plants of indeterminate habit, fruiting interferes with the stem elongation. In cases like *Datura metel* where fruit setting continues for a long time, stem elongation and formation of new shoot continue for some time more even after the fruits have formed; this is the case in tomatoes as well. If exfloration is practiced on such plants vegetative growth will continue indefinitely, as shown by the results obtained in this investigation.

In tomatoes, Murneek (5, 6) observed that fruit production limits growth and exfloration or de fruiting increases the size of the plant. In soybean, a short-day plant of indeterminate habit, no increase in vegetative development, following exfloration, was noted by Austin (11). Such a differential behavior of the soybean is attributed to its photoperiodic properties. In this case, shortening of day length not only initiates the reproductive phase, but also curtails its vegetative processes. *Datura metel*, though not exactly a long-day plant, has been found to show greater vegetative growth with longer exposures of light than with shorter duration of illumination (15). Its behavior is, therefore, like that of tomatoes which when exflorated continue vegetative development indefinitely.

SUMMARY

The effect of pruning and exfloration on the growth and alkaloidal content of *Datura metel* has been described. The results indicate that heavy pruning brings about a severe injury to the whole plant and impairs its vigor so much that the plant

is not able to make good this loss until late in the life cycle. Stem height, leaf number, and dry weight are significantly depressed. Mild pruning does not affect the plant so adversely, and the pruned plants approach the control ones in their vegetative growth.

Exfloration brings about an all-round increase in these directions. Height, leaf number, and dry matter production are all significantly increased. This beneficial effect is marked even on partly exflorated plants.

On alkaloid content exfloration shows a significant effect. Pruning has no effect in this regard. Greatest yield in dry matter and alkaloid content is obtained in plants which are early exflorated and also treated with nitrogen before exfloration. Plants exflorated later or those that are partially exflorated are not benefited much in these regards.

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Observations on the Leaves of Several Species of Viburnum*

By HEBER W. YOUNGKEN†

The results of a comparative study of the leaves of *Viburnum prunifolium, V. rufidulum,* and *V. cassinoides* are reported. Determinations of the vein islet numbers, palisade ratios and stomatal indexes of the three species are included.

IN A PAPER dealing with the history, botany, and pharmacognosy of four species of *Viburnum* (1), the author discussed the characteristics of the plants and barks of *Viburnum prunifolium, V. rufidulum, V. cassinoides,* and *V. nudum.* At that time the medicinal properties of these Viburnums were only known to occur in the bark. Since that time extracts of the leaves of several species have been reported (2) to possess properties similar to that of the barks. If further research confirms this report, the leaves may be found more economical sources of the Viburnum drugs, and their collection instead of the bark tends to conserve the natural stands of these *Viburnum* species which have undergone depletion for many years. The purpose of this paper is to report the writer's more detailed observations made on the leaves of *Viburnum prunifolium* L., *Viburnum rufidulum* Raf., and *Viburnum cassinoides* L.

MATERIALS

The leaves of the *Viburnum* species described in this paper were gathered from authenticated plants growing in the Arnold Arboretum and Medicinal Plant Garden of the Massachusetts College of Pharmacy at Jamaica Plain, Mass., during 1943–1947 and from herbarium sheets and preserved specimens of leaves gathered by the writer in North Carolina and Tennessee during his collecting trips in the South previous to World War II.

PHYSICAL CHARACTERISTICS OF LEAVES

Viburnum prunifolium.—Leaves petiolate, 2.5 to 7 cm. in length and 1.2 to 3 cm., occasionally up to 5 cm. in width; lamina ovate, oval, or suborbicular, its apex rounded or acute, its base cuneate or rounded, its margin serrulate to occasionally serrate in places, and with incurved, callous-tipped teeth; upper surface dark green, sometimes tinged and spotted with purple and red, glabrous; lower surface pale green and glabrous or with rusty hairs along the midrib and in the axils of the secondary

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veins; venation pinnate-reticulate; texture subcoriaceous to coriaceous at maturity; petiole short, slender, grooved above, green to purplish, 0.2 to 1.6 cm. in length, occasionally narrow winged, especially on strong shoots. As shown by Sargent (3) and also observed by the writer, the amount and nature of the pubescence on these leaves varies. In the northern United States the leaves are usually glabrous, while in the southern United States the under surface is frequently marked by the presence of numerous rusty hairs. Odor, valerian-like, on crushing or moistening; taste astringent and bitter.

Viburnum rufidulum.—Leaves petiolate, 4 to 10 cm. in length and up to 6 cm. in width; lamina ovate, elliptic to elliptic-obovate, usually obtuse but sometimes rounded or emarginate at apex, serrulate or denticulate along the margin, the teeth callous-tipped and frequently only slightly incurved, rounded, cuneate, or inequilateral at base; upper surface dark green, lustrous, and glabrous; lower surface pale green, rusty pubescent toward the base, venation pinnate-reticulate; texture coriaceous; petiole narrowly to broadly winged, 6 to 12 mm. in length, rusty-pubescent, especially beneath; odor valerian-like on crushing or moistening; taste astringent and bitter.

Viburnum cassinoides.—Leaves petiolate, the lamina elliptic, ovate, ovate-elliptic, ovate-lanciolate or oblong, occasionally oblanceolate, 3 to 10 cm. in length, and up to 4.5 cm. in width, the apex abruptly acuminate or obtuse, the base cuneate or rounded, the margin undulate or crenate to obscurely dentate. The upper surface dull green, nearly glabrous, the lower surface pale green, more or less scurfy on the veins; petioles 1 to 2 cm. in length, odor valerian like; taste astringent and bitter

HISTOLOGY OF LEAVES

Viburnum prunifolium.—Transverse sections of the lamina outside of the midrib exhibited dorsiventral structure with an upper epidermis of tangentially elongated epidermal cells possessing a moderately thickened cuticle, a palisade parenchyma of a single layer of cells occupying about one-third of the width of the section, spongy parenchyma traversed by fibrovascular tissue, and a lower epidermis similar to that of the upper epidermis except for a somewhat thinner cuticle and the presence of stomata.

Cross sections of the midrib were convex above and below and showed an epidermis with capitate and stellate hairs. In the center occurred a meristele with narrow collateral fibrovascular bundles separated by medullary rays 1 cell in width. A pericycle surrounded the bundles which, excepting in young leaves, possessed pericyclic fibers arranged in the form of an arc together with few isolated fibers below the meristele. The region between the epidermis and pericycle was occupied by collenchyma and parenchyma, some of the cells of which contained rosette aggregates of calcium oxalate. The secondary veins were devoid of pericyclic fibers.

Surface sections of the lamina showed an upper epidermis with epidermal cells having slightly curvilinear vertical walls, and no stomata and a lower epidermis with epidermal cells having pronounced curvilinear vertical walls. Numerous oval to broadly oval stomata occurred in this epidermis. No regularity occurred in the arrangement of the subsidiary cells around the guard cells of the stomata. Frequently 5 to 6 occurred and, occasionally, one or two elongated crescent-shaped subsidiary cells were found parallel to the guard cells.

Both epidermises possessed small capitate hairs and stalked stellate hairs. The lower epidermis of young leaves showed short, 1- to 2-celled, simple nonglandular hairs, the latter with thick wall and narrow lumen.

Transverse sections of the petiole exhibited three vascular strands imbedded in parenchyma, the latter surrounded by collenchyma, scattered cells in the parenchyma containing rosette aggregates of calcium oxalate. An epidermis bearing capitate and stellate hairs covered the exterior. Some of the epidermal cells contained anthocyanin.

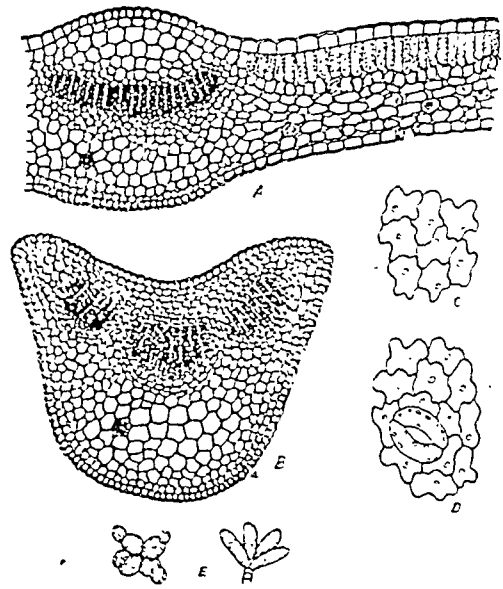
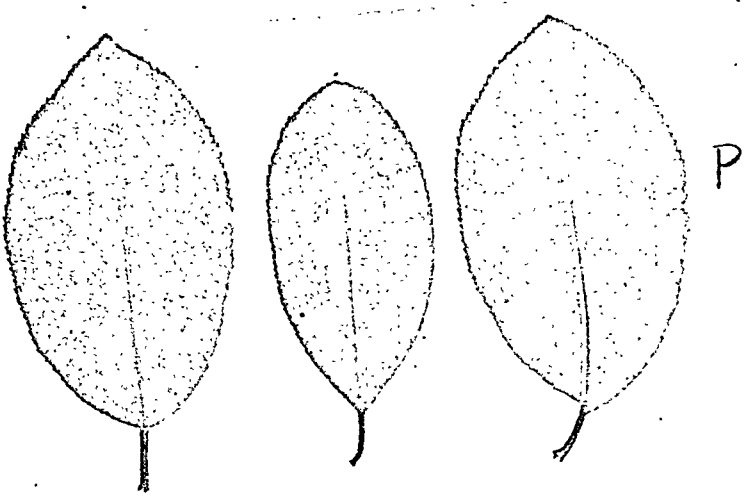


Fig. 2.—*Viburnum prunifolium* Linné. Lea Histology. *A*, transverse section of lamina showing a secondary vein and interneural region; *B* transverse section of petiole; *C*, upper epidermis *D*, lower epidermis; *E*, hairs.

Viburnium rufidulum.—Transverse sections o the lamina outside of the midrib exhibit dorsiventral structure. The upper epidermis possesses tangentially elongated epidermal cells with a thick cuticle. The palisade parenchyma consists of a single layer of richly chlorophylloid cells occupying about one-fourth of the depth of the mesophyll. The spongy parenchyma consists of irregularly lobed cells and is traversed by fibrovascular tissue. The lower epidermis shows many stomata and its cuticle is somewhat thinner than that of the upper epidermis. Both epidermises show stellate hairs with short and long stalks and short-stalked peltate hairs, especially numerous on young leaves.

FIG. 1.—Viburnum Leaves. P, *Viburnum prunifolium* Linné; R, *Viburnum rufidulum* Rafinesque; C, *Viburnum cassinoides* Linné. X7/8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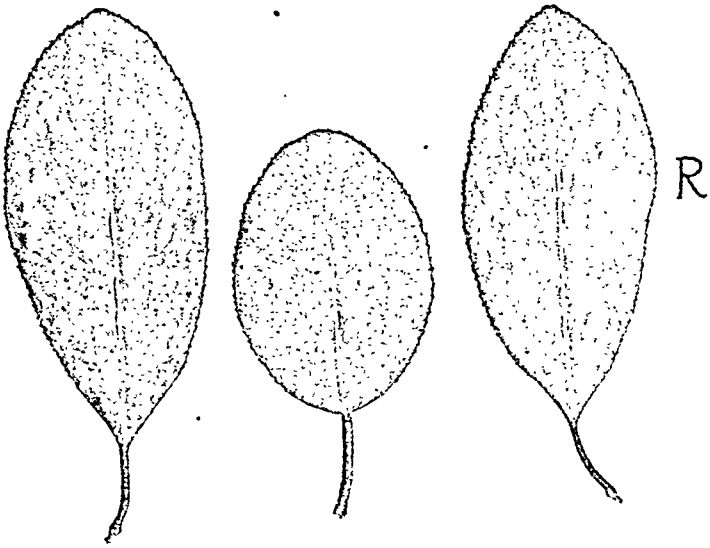
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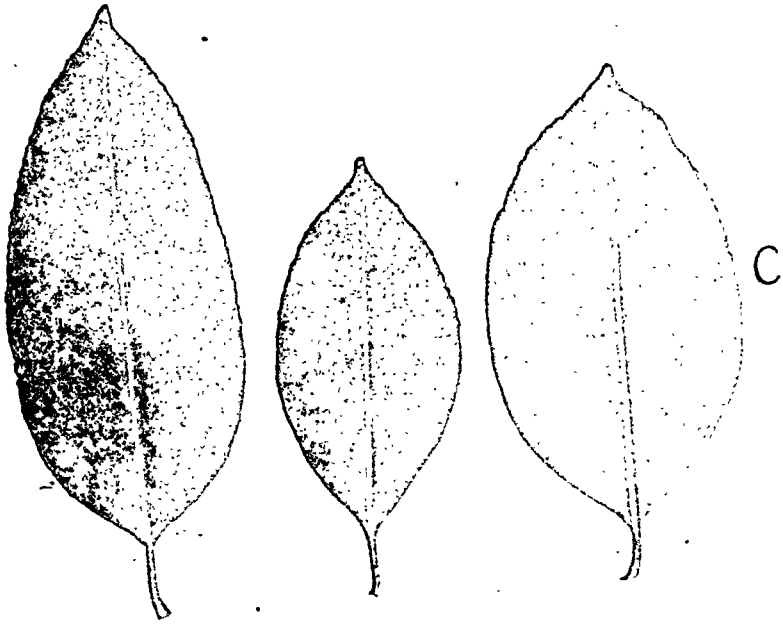
C

Cross sections of the midrib are convex above and below. The central region shows a crescent-shaped meristele of narrow collateral fibrovascular bundles separated by medullary rays one cell in width. A pericycle surrounds the bundles which, in the region beneath the meristele, contain an arc of pericyclic fibers, while a few pericyclic fibers occur above the meristele.

Surface sections of the lamina show the epidermises to be somewhat similar to those of *V. prunifolium*, the cells being curvilinear in outline.

The stomata of mature leaves studied were 41.8 to 53.2 μ in length. The petiole possesses a large crescentic and 2 small circular strands of fibrovascular tissue. On the lower face of the crescentic meristele occurs a thick arc of pericyclic fibers.

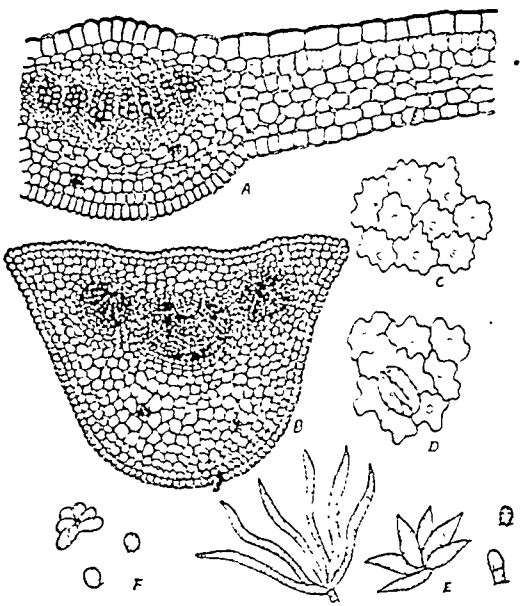

Fig. 3.—*Viburnum rufidulum* Raf. Leaf Histology. *A*, transverse section of lamina showing midrib and interneural region; *B*, transverse section of petiole; *C*, upper epidermis; *D*, lower epidermis; *E*, *F*, hairs.

Viburnium cassinoides.—Transverse sections of the lamina exhibited the following structures: upper epidermis of a layer of tangentially elongated epidermal cells with thick cuticle, palisade parenchyma of a single layer of chlorophylloid cells occupying about two-fifths the depth of the section, spongy parenchyma of irregularly lobed cells and air-spaces, the region traversed by fibrovascular tissue, lower epidermis with stomata and moderately thickened cuticle.

Surface sections of the upper epidermis showed epidermal cells with moderately curvilinear vertical walls. Surface sections of the lower epidermis exhibited epidermal cells with a striated cuticle whose vertical walls were pronouncedly curvilinear, and numerous oval to elliptic stomata. Both epidermises possess many short-stalked, peltate hairs, the shield of which usually has 8 cells, and some small capitate hairs.

Cross sections of the midrib are convex above and below. In the center is a crescent-shaped meristele of narrow bundles separated by medullary rays 1-cell in width. No pericyclic fibers occurred in the sections studied. Most of the cells between the meristele and lower epidermis represented collenchyma, while the region above the meristele consisted largely of parenchyma with 2 or 3 layers of collenchyma directly beneath the upper epidermis.

Rosette aggregates of calcium oxalate up to 39 μ in diameter occurred in the parenchyma and collenchyma of the midrib and petiole. As in the petioles of the two other Viburnums discussed in this paper, the petiole of *V. cassinoides* showed 3 fibrovascular strands and its epidermis possessed the same kinds of hairs as the lamina.

VEIN ISLETS

The vein islets of three leaves of each of the aforementioned *Viburnum* species were counted in each of five fields in cleared strips cut from the apex, base, and between the midrib and margin in the mid region of each leaf (margin-center), and the vein islet numbers ascertained by the method of Zufall and Burlage (4) with slight modification. These values are shown in the following tables.

TABLE I.—VIBURNUM PRUNIFOLIUM LEAF APEX[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	3.5	3.5	3.5
2	4.0	3.5	3.5
3	3.5	4.0	4.0
4	3.5	3.0	3.0
5	4.0	4.0	4.0

[a] Average vein islet no. for apex 3.63.

TABLE II.—VIBURNUM PRUNIFOLIUM LEAF BASE[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	3.0	3.5	3.5
2	3.5	4.0	3.0
3	4.0	4.0	4.0
4	3.5	3.5	3.5
5	4.5	3.0	3.0

[a] Average vein islet no. for base 3.56.

TABLE III.—VIBURNUM PRUNIFOLIUM LEAF MARGIN-CENTER[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	3.0	3.0	3.0
2	3.0	3.0	3.0
3	3.0	3.0	2.5
4	3.0	3.0	3.0
5	4.0	2.5	4.0

[a] Average vein islet no. for margin-center is 3.06. Average vein islet number for leaf of *Viburnum prunifolium* is 3.41.

TABLE IV.—VIBURNUM RUFIDULUM LEAF APEX[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	4.0	4.0	3.5
2	5.0	4.5	3.5
3	3.5	4.5	4.5
4	3.5	4.0	4.5
5	4.5	4.0	4.5

[a] Average vein islet no. for apex is 4.13.

TABLE V.—VIBURNUM RUFIDULUM LEAF BASE[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	3.5	4.0	3.0
2	3.5	4.5	3.0
3	4.0	4.0	3.0
4	3.0	4.5	3.5
5	4.0	3.5	4.0

[a] Average vein islet no. for base is 4.1.

TABLE VI.—VIBURNUM RUFIDULUM LEAF MARGIN-CENTER[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	4.0	4.0	4.0
2	4.0	4.0	5.0
3	4.0	4.5	4.0
4	4.0	4.5	5.0
5	4.5	4.0	3.5

[a] Average vein islet no. for margin-center is 4.2. The average vein islet number for leaf of *Viburnum rufidulum* is 4.13.

TABLE VII.—VIBURNUM CASSINOIDES LEAF APEX[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	3.5	3.0	3.5
2	3.5	3.5	3.0
3	3.5	3.0	3.5
4	4.0	3.5	3.5
5	3.5	2.5	3.5

[a] Average vein islet no. for apex is 3.36.

TABLE VIII.—VIBURNUM CASSINOIDES LEAF BASE[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	3.5	5.5	3.5
2	3.5	5.5	3.0
3	3.0	3.5	3.0
4	5.0	4.5	3.5
5	4.0	4.5	3.5

[a] Average vein islet number for base is 4.

TABLE IX.—VIBURNUM CASSINOIDES LEAF MARGIN-CENTER[a]

Field	Leaf 1	Leaf 2	Leaf 3
1	4.0	3.5	3.5
2	4.0	3.5	3.5
3	4.5	4.0	5.0
4	4.0	4.0	3.0
5	4.0	3.5	3.5

[a] Average vein islet number for margin center is 3.96. The average vein islet number for the leaf of *Viburnum cassinoides* is 3.77.

STOMATAL INDEX

The stomatal index of the leaves of *Viburnum prunifolium*, *V. rufidulum*, and *V. cassinoides* was determined by removing strips of the lower epidermis of these leaves, mounting in 10 per cent of glycerin solution, counting the number of stomata and epidermal cells separately in 1 mm. square areas and applying the formula of Salisbury (5). The Whipple ocular micrometer, 4 mm. objective, and 10× ocular were employed in the counting technique. The stomatal index of one leaf of *Viburnum prunifolium* was found to be 12.6; of one leaf of *Viburnum rufidulum*, 12.27; of one leaf of *Viburnum cassinoides*, 13.3. Further determinations on additional leaves of these species are in progress.

PALISADE RATIO OF VIBURNUM LEAVES

A preliminary study was made of the palisade ratios of the leaves of *Viburnum prunifolium*, *V. rufidulum*, and *V. cassinoides*. The method followed was to cut pieces 2–3 mm. square by means of a sharp blade from the apex, margin-center, and base. The segments were placed in clearing solution consisting of 50 Gm. chloral hydrate dissolved in 20 cc. of water and gently heated for 15 minutes. The leaf pieces were then mounted in Berlese's Mountant Modified and examined under the compound microscope, using high power magnification. In each determination, the number of palisade cells under any 4 adjacent epidermal cells was divided by four, the quotient representing the palisade ratio. The results of this study for one leaf of each of the three species are as follows:

PALISADE RATIOS

Vibrunum prunifolium.—Apex, 3.2. Margin-center, 3. Base, 3. Average, 3.06.

Viburnum rufidulum.—Apex, 3.2. Margin-center, 2.7. Base 3. Average, 2.96.

Viburnum cassinoides.—Apex 3. Margin-center 3. Base, 2.5. Average, 2.83.

Further studies on the palisade ratios of these species are anticipated.

SUMMARY

1. The leaves of *Viburnum prunifolium*, *Viburnum rufidulum*, and *Viburnum cassinoides* are described as to their physical characteristics and illustrated by photographs.

2. The histology of each of these leaves is described in detail with accompanying drawings illustrating the histology of the lamina and petiole.

3. The average vein islet number values have been determined for each of these leaves. They are as follows: for *Viburnum prunifolium*, 3.41; for *Viburnum rufidulum*, 4.13; for *Viburnum cassinoides*, 3.77.

4. A preliminary report is given of the stomatal indexes and palisade ratios determined by the author for leaves of the aforementioned species. Additional work on these is in progress.

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Critical Aspects of an Acidic Germicidal Lotion*

By G. L. CHRISTENSON and ROBERT S. SHELTON†

Experiments on desirable modifications of an acidic germicidal lotion are described. The basic emulsification system of water phase, pectin, cetyl trimethyl ammonium bromide, and oleaginous phase is so modified as to improve shelf storage stability, enhance germicidal activity, decrease irritation, and improve emolliency. Critical physical, bacteriological, and pharmacological effects of ingredients such as pectin type and level, mineral oil and lanolin type and level, and bodying agents are discussed.

THE OBJECT of these studies was to determine the effect of various factors on the physical and bacteriological properties of an acidic germicidal lotion. As a result of previous work in our laboratories (1, 2) on the bactericidal efficacy of quaternary ammonium compounds and the use of such compounds as adjuncts to pectin as coemulsifying agents, a basic formula was chosen essentially as outlined in Table I.

TABLE I.—BASIC FORMULA FOR GERMICIDAL LOTION

Mineral oil	12.00%
Lanolin (Anhydrous)	1.00
Pectin	1.00
Boric Acid	2.00
Cetyl Trimethyl Ammonium Bromide	0.16
Perfume	0.10
Distilled Water, *q. s.*, to make	100

Our experiences with this basic formula had indicated that the use of pectin with the quaternary ammonium compound, cetyl trimethyl ammonium bromide, resulted in a considerable increase in emulsification efficacy over pectin alone; thus substantiating the claims of Manchey and Schneller (3), but certain properties of the proposed emulsion necessitated this further study. The qualities which we were desirous of improving were: long-term shelf stability, germicidal activity, emolliency, corneal irritation, and odor stability.

EXPERIMENTAL

Emulsion Preparation.—Emulsions were made by preparing the water and oleaginous phases separately and combining with high-speed stirring. Emulsification was concluded by homogenization, consisting of passage through one of three machines: laboratory hand homogenizer, Manton Gaulin homogenizer, or Kom-bi-nator. Comparative studies were made on the Continental and English methods of combination and inasmuch as no significant difference in the final product was discernible, the Continental method was chosen because of its adaptability to plant operations. In this method the oleaginous ingredients were melted together at the lowest possible temperature, and the pectin was dispersed therein. This pectin-oil dispersion was then added to the water phase. The resulting emulsion was agitated continuously until the pectin was completely hydrated, after which the preparation was homogenized.

Centrifuge Stability.—Samples were then subjected to an accelerated centrifuge test consisting of rotation of sample at 3600 r. p. m. in a 10-inch angle head centrifuge for one hour. Attempts were made to measure the per cent of creaming as induced by this treatment, but because of the varying demarcation of cream layer, it was impossible to measure accurately the more stable preparations. This procedure does eliminate the more unstable samples and its use in this regard was valuable.

Storage Conditions.—Samples which survived the centrifugation test were stored at the various temperatures routinely employed in our product development studies (4°, room temperature, 37°, and 45°). These stored samples were examined at weekly intervals for the first month and thereafter at six-month and one-year intervals. From the sales and distribution standpoint, we feel that a product of the liquid emulsion type should show no change in physical properties for at least one year at room temperature and 4°. Slight creaming is allowable at 37° at the six-month period if no change in room temperature samples occurs in one year. Samples must be stable at 45° for at least one month and meet the foregoing requirements to be considered stable. These are rigid standards, but past experience has shown that they are minimal for a product of this nature to maintain its elegance under all distributional conditions.

Viscosity Determination.—Viscosity of preparations was determined by a pipette method, and is recorded as viscosity relative to water. For these studies the tip of a 10-cc. pipette was cut off and fire-polished so that the water delivery time was 2.8 seconds. The time required for emptying of a given sample, divided by the water delivery time, is the relative viscosity. No attempt was made to apply the minor corrections involved, but since these determinations were all made at 30°, and since the viscosity of water at 25° is 0.00805 poises, for all practical and comparative purposes one relative viscosity unit is equal to one centipoise unit.

Oil Globule Determination.—Oil globule counts were made by mixing an accurately weighed sample with known volume of melted 5% gelatin solution and spreading a measured amount of this dispersion over an area of exactly 1 sq. cm. on a microscope slide. This slide is allowed to harden in the

* Received March 26, 1948, from the Pharmaceutical Research Department, Research Laboratories, The Wm. S. Merrell Company, Cincinnati, O.

† We gratefully acknowledge the assistance of the Analytical, Bacteriology, and Pharmacology Departments in conducting the oil globule count, germicidal and corneal irritation evaluations, and of H. J. Long in the preparation of the figure.

refrigerator to retard Brownian movement and is then examined at 600 × magnification with a calibrated eyepiece. By averaging the number of globules per given area in various portions of the field an accurate transposition of number of globules per mg. of original emulsion can readily be made.

Germicidal Evaluation.—Critical Killing Time determinations were conducted in inoculating the lotion, "as is," with vegetative growth of the indicated test organism. Unless otherwise indicated, *Staphylococcus aureus* was employed as the test organism. Subcultures were then made from the inoculated lotion into "Letheen" broth after the indicated time intervals to inactivate any residual quaternary ammonium compound (4). Results of duplicate tests are recorded. Himebaugh cup determinations, consisting of measurement of zone of inhibition of growth of test organism in agar media, were also made. In view of the work of Quisno and Foter (5) on the decrease of germicidal activity of quaternary ammonium compounds by agar, we do not consider these data indicative of the true activity of quaternary ammonium compounds, and no attempt is made to correlate them with the other data reported.

RESULTS

As indicated previously, no difference was found in formulation of the basic formula by Continental or English methods. The method of homogenization did cause significant differences in emulsion quality. The differences in homogenization efficiency of three types of homogenization tools are shown in Table II. These data show that the hand laboratory homogenizer is better than no homogenization, but is not comparable to the two commercial machines employed. The Kom-bi-Nator is remarkably efficient as a homogenizing machine for this type of preparation.

A number of different types of pectin were employed in the basic formula as shown in Table III. From these data it is evident that there is considerable difference in pectins as co-emulsifiers for this basic emulsion. In two cases it was not possible to obtain satisfactory emulsions initially. Low-methoxy pectin resulted in satisfactory emulsification, but because of the thinness of preparation, some creaming occurred in six months. Stability of preparations containing the 210C No. 8 and 100 gel strength pectins was similar to that of the low-methoxy pectin preparations. All three lots of Pectin N. F. employed produced a satisfactorily stable emulsion. The data shown in this table also are illustrative of the previously mentioned lack of complete correlation between the accelerated centrifuge stability test and long-term shelf stability results.

TABLE II.—EFFECT OF TYPE OF HOMOGENIZER ON EMULSION QUALITY

Type Homogenizer	Pressure Employed	Oil Globule Count, Millions per Mg.
None	.....	6
Hand Homogenizer[a]	?	55
Manton Gaulin[b]	2000 lbs. per sq. in.	380
Kom-Bi-Nator[c]	750 lbs. per sq. in.	1323

[a] Hand model—American Profession Pharmacist.
[b] Type 25CGB—Manton Gaulin Mfg. Co.
[c] Model K-200—Marco Co., Inc.

We were further interested in studying the effect of variation of certain ingredients in the basic formula on viscosity and Critical Killing Time of the lotion. In Table IV the effect of variation of mineral oil-lanolin levels and pectin levels on relative viscosity and Critical Killing Time is shown. The changes due to variation of pectin level are in the order expected—an increase in viscosity and no difference in Critical Killing Time. However, in the case of increasing levels of oleaginous phase, rather surprising increases in viscosity were encountered along with unexpected prolongation of Critical Killing Time. We have hypothesized that this phenomenon is a function of alteration of the degree of emulsification. The abnormal rise in viscosity is attributed to an increased rigidity at the globule interfaces and the decrease in Critical Killing Time to inaccessibility of the quaternary ammonium compound because of its tie-up at the globule interfaces. The effect is presented in somewhat clearer fashion in Fig. 1.

TABLE III.—EFFECT OF VARIOUS PECTINS ON EMULSION PROPERTIES

Pectin Employed	Relative Viscosity	Critical Killing Time, Min.	Centrifuge Stability	Shelf Stability
Low Methoxy—Citrus	2.0	3–5	OK	Slight creaming at six months
Pectin NF—Lot A	9.1	3–5	OK	OK at one year
Pectin NF—Lot B	9.0	3–5	OK	OK at one year
Pectin NF—Lot C	8.2	5	OK	OK at one year
100 Gel Strength—Apple	1.5	3–5	Creamed excessively	Creamed and separated at one month
150 Gel Strength—Apple	Emulsion very dark and thin—separated readily—no further study made			.....
Rx Grade—No. 300—Apple	Emulsion curdled—unsatisfactory			.....
Rx Grade—No. 3—Na Salts—Apple	5.5	10	OK	Creamed and separated at six months
210C No. 8—Apple	5.8	10	OK	Slight creaming at one month, creamed at six months

Since we were interested in retaining germicidal activity while increasing stability, viscosity, and emolliency, the data here appeared to preclude achievement of all goals. Various adjuncts to the oleaginous phase such as glyceryl mono-stearate glyceryl mono-oleate, hydrogenated vegetable oils, and polyoxyalkalene derivatives were tested without achieving all of the desired goals. Attempts to modify the water phase by polyethylene glycols resulted in undesirable emulsion characteristics but the use of methyl cellulose as a bodying agent gave promising results. It was found that although methyl cellulose alone would not satisfactorily replace pectin in the formulation, its use as an adjunct to pectin resulted in increased viscosity without detracting from the desired bacteriological qualities. With the aid of this agent we were able to make preparations of any desired viscosity.

At the time this work was done, the quality of lanolin commercially available was extremely variable. Studies on various grades of lanolin revealed that the more highly purified grades were the most satisfactory. Apparently, impurities in regular anhydrous Lanolin U. S. P. were instrumental to some degree in inactivation of the germicidal action of cetyl trimethyl ammonium bromide. Cosmetic grade lanolin was selected as free from this inactivation phenomenon and the effect of varying levels of this material in the formulation was tested. As shown in Table V, levels of 2.4% to 3.6% of this material were optimal in so far as emulsion stability and Critical Killing Time were concerned. Increase in concentration of this material also resulted in improvement of emolliency, a quality which is difficult to express objectively. Preparations were tested comparatively by application of equal amounts to a 4-sq. in. skin area, and observing effects at application and after fifteen- and thirty-minute intervals. The bland aroma of this material also resolved, to a considerable degree, the question of perfume perception and stability which was initially a problem.

The basic formula was initially submitted to our Pharmacology Department for rabbit corneal irritation tests. This test involves direct application to

TABLE IV.—EFFECT OF VARIATION OF PECTIN AND OIL ON LOTION CHARACTERISTICS

Sample	Mineral Oil/Lanolin Ratio	Pectin/Cetyl Trimethyl Ammonium Bromide Ratio	Water/Pectin Ratio	Oil/Pectin Ratio	Pectin, %	Mineral Oil Lanolin, %	Relative Viscosity	Critical Killing Time, Min.
A	11.7	6.4	87.7	9.0	1.0	9.0	4.4	10–20
B	11.7	6.4	81.8	14.0	1.0	14.0	5.9	20
C	11.7	6.4	78.8	18.0	1.0	18.0	7.6	20
D	11.7	6.4	72.8	24 0	1.0	24.0	22.4	20–30
E	11.7	6.4	60.8	36.0	1.0	36.0	52.4	60
F	11.7	5.0	106.0	15.7	0.8	12.6	2.7	10–20
G	11.7	6.0	94.8	14.0	0.9	12.6	4.6	10–20
H	11.7	6.2	85.2	12.6	1.0	12.6	5.6	10–20
I	11.7	7.5	71.0	10.5	1.2	12.6	7.9	10–20
J	11.7	8.9	60.7	9.0	1.4	12.6	9.5	10–20

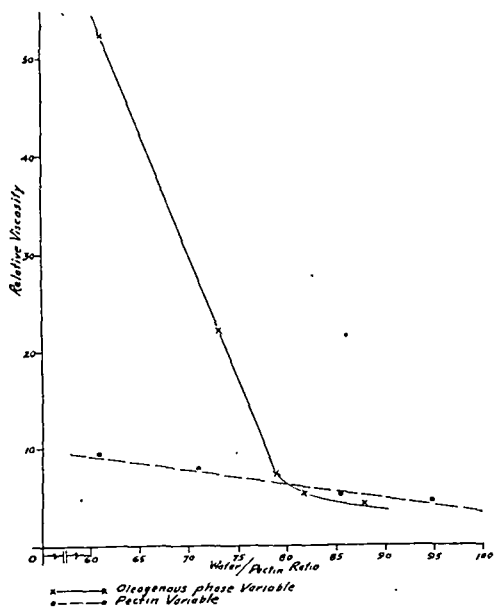

Fig. 1.—Effect of Variation of Oil and Pectin on Relative Viscosity.

TABLE V.—EFFECT OF VARIATION IN LANOLIN LEVEL ON LOTION PROPERTIES

% Lanolin (Cosmetic Grade)	Critical Killing Time, Min.	Stability
None	<3	Curdled in three weeks at room temperature
0.3	3	Curdled in one month at room temperature
0.6	<3	Slight creaming, curdled in six months at 37°. OK for one year at room temperature
1.2	3	Slight creaming, curdled in six months at 37°. OK for one year at room temperature
2.4	<3	OK for one year at all temperatures
3.6	3	OK for one year at all temperatures
4.8	<3	Slight creaming in one year at room temperature

TABLE VI.—GERMICIDAL EFFICIENCY OF BABY LOTION

Organism	No Serum 3′	5′	10′	20′	30′	60′	10% Added Serum 3′	5′	10′	20′	30′	60′
Staphylococcus aureus	++	−−	−−	−−	−−	−−	++	+−	−−	−−	−−	−−
Eberthella typhosa	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−
Streptococcus viridans	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−
Staphylococcus albus	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−
Streptococcus hemolyticus	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−
Escherichia coli	++	++	+−	−−	−−	−−	++	++	++	++	+−	−−
Pseudomonas aeruginosa	++	++	−−	−−	−−	−−	++	++	++	++	−−	−−

+ Indicates growth of the organism after subculturing.
− Indicates no growth of the organism after subculturing.

the cornea and is an acute and severe test; but since our aim was to produce a satisfactory baby lotion, we felt that little irritation should be manifest in this severe test in view of the proposed application to the tender skin of the newborn infant. The basic lotion was severely irritating as measured by rabbit corneal application. After embodiment of several changes in the formulation (methyl cellulose incorporation, mineral oil increase, lanolin increase and change in quality, and modification of perfume) samples were resubmitted and found to exert very slight, if any, transient irritation.

Also, after incorporation of the changes previously described, the lotion was submitted to the Bacteriology Department for germicidal evaluation against a number of organisms commonly encountered on the skin. Their results are shown in Table VI. These results indicate that the lotion possesses remarkably good germicidal properties and, in view of its intermittent periodic application, might reasonably be expected to keep the bacterial flora of the skin surface to which it is applied at a minimum.

Clinical studies have further substantiated our belief that the emulsion system results in a therapeutically efficacious, mild, and soothing lotion. Preliminary studies (6) have indicated that it is an excellent agent for prevention and cure of impetigo epidemics among newborn infants.

SUMMARY

Experiments on desirable modifications of an acidic germicidal lotion are described. The basic emulsification system of pectin and cetyl trimethyl ammonium bromide is modified to improve shelf storage stability, enhance germicidal activity, decrease irritation, and improve emolliency. Critical aspects of the various ingredients such as pectin type and level, mineral oil and lanolin type and level, and bodying agents are discussed.

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The Turbidimetric Determination of Peptic Activity*

H. J. WESSELMAN and W. W. HILTY†

The use of crystallized egg albumen as a new substrate is described in a rapid method for the determination of peptic activity. The over-all time consumed in the assay of a series of pepsin samples represents approximately one-third of the time that is required for the officially described N. F. procedure. The accuracy is improved by this method and the variations in the substrate that have been so frequently observed are practically eliminated.

BECAUSE of the variation in the egg albumen used in the standardization of pepsin preparations as directed in the National Formulary VIII (1), it has not always been possible to obtain good digestion of the substrate and thus it has been very difficult to determine the relative strength of pepsin preparations from time to time. There are many factors which could account for this variation, none of which can be controlled satisfactorily by the analyst interested in determining the potency of the preparations in question.

A number of approaches to the standardization of pepsin were considered. However, due to the complexity of the compound and the nature of its action, the most logical procedure still remains that of digesting some form of protein. Based on this assumption our efforts were then directed toward the development of a substrate

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which would eliminate most of the variable factors.

The literature contains many references (2–6) on determining pepsin in body fluids and in these a number of methods of approach are described. There are also various methods reported for crystallizing egg albumen. This appeared to open the way for the preparation of a more stable and more uniform substrate than has previously been used.

EXPERIMENTAL

A crystallized egg albumen was prepared in accordance with the method suggested by Cole (7). The solution thus obtained was further treated by freezing and then drying in a vacuum. This preparation is very soluble in water and a 1% solution when heated in a boiling water bath for fifteen minutes to coagulate the protein yields a very uniform and finely divided suspension. This suspension can be reproduced and is stable for periods of at least one month when stored in a refrigerator.

The availability of the uniform and finely divided suspension suggested the possibility of determining the unused portion of the substrate by a photoelectric measurement of the turbidity of the digested sample. To accomplish this it was necessary to determine whether or not the rate of digestion would proceed in a uniform manner and if the reaction could be terminated for a sufficient length of time to allow an accurate reading to be taken. Results as shown in Table I and illustrated graphically in Fig. 1 indicate that the reaction does proceed uniformly for approximately fifty minutes and from those shown in Table II and in Fig. 2 it is observed that the immediate chilling of the reaction mixture will completely retard the peptic action for at least one-half hour. This is ample time to permit the necessary readings on an instrument equipped to make turbidimetric readings.

TABLE I.—RATE OF DIGESTION DETERMINED BY INCREASE IN GALVANOMETER READING PER UNIT TIME

Time, Min.	A 3.33 Mg. N. F. Reference Pepsin	B 3.33 Mg. N. F. Reference Pepsin	C 3.33 Mg. Pepsin 1:3000	D 3.33 Mg. Pepsin 1:3000
0	34.5	34.5	34.5	34.5
5	38.0	38.0	38.0	38.0
10	40.0	40.0	40.0	40.0
15	42.5	41.5	42.5	42.5
20	44.0	43.0	45.0	44.5
25	46.5	45.0	47.5	47.5
30	50.0	47.5	52.5	51.5
35	52.0	51.5	56,5	55.0
40	56.5	56.0	61.0	60.0
45	60.0	59.5	68.0	66.0
50	67.0	64.0	78.5	76.0
55	73.0	70.0	89.5	85.5
60	81.0	77.0	+100.0	99.0

Reagents

(1) *0.3% HCl.*—Mix 35 cc. of 1 *N* hydrochloric acid with 385 cc. of distilled water.

(2) *Albumen Substrate.*—Prepare a crystallized egg albumen as suggested by Cole (7). Further treat the solution thus obtained by freezing and then drying in a vacuum. Dissolve 1.0 Gm. of crystalline egg albumen in 100 cc. of distilled water. Heat in boiling water bath for fifteen minutes with constant agitation. Strain through muslin into 100-cc. volumetric flask, cool, and add sufficient distilled water to make 100 cc.

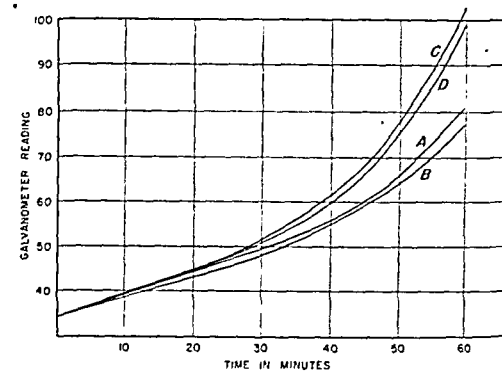

Fig. 1.—Rate of digestion determined by increase in galvanometer reading per unit time.

TABLE II.—EFFECT OF CHILLING REACTION MIXTURE

Time, Min.	A 3.33 Mg. N. F. Reference Pepsin	B 3.33 Mg. Pepsin 1:3000	C 2.86 Mg. Pepsin 1:3000
52° C.			
0	34.5	34.5	34.5
45	60	55	48
Ice Bath			
5	60	57	49
15	60	56	49
30	60.5	56	49.5
45	60	56	49.0
55	60	56	49.0

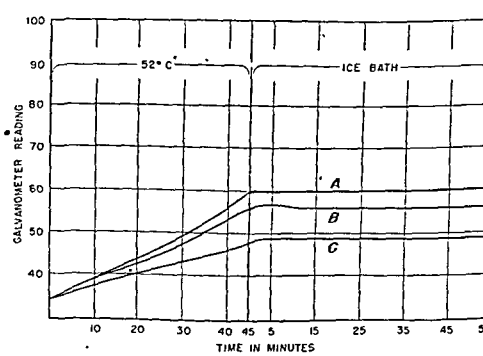

Fig. 2.—Effect of chilling reaction mixture.

(3) *N. F. Reference Pepsin.*—(A) Dissolve 0.1000 Gm. of N. F. Reference Pepsin in sufficient 0.3% HCl to make 100 cc.

(B) Transfer 10 cc. of solution A to a 100-cc. volumetric flask and dilute to 100 cc. with 0.3%

TABLE III.—COMPARISON OF RESULTS

Sample	N. F. VIII Method Standard Cc. Residue	N. F. VIII Method Sample Cc. Residue	N. F. VIII Method Relative Strength	Proposed Method Standard Reading	Proposed Method Sample Reading	Proposed Method Relative Strength
A						
1:3000	18.5	15.0	+1:3000	60	63.0	+1:3000
1:3500	18.5	18.0	+1:3500	60	54.0	−1:3500
B						
1:3000	18.5	14.0	+1:3000	60	61.0	+1:3000
1:3500	18.5	19.0	−1:3500	60	55.0	−1:3500
C						
1:3000	18.5	12.0	+1:3000	60	63.0	+1:3000
1:3500	18.5	18.0	+1:3500	60	56.5	−1:3500
D						
1:3000	18.5	15.0	+1:3000	60	63.5	+1:3000
1:3500	18.5	21.0	−1:3500	60	58.0	−1:3500
E						
1:3000	27.0	26.0	+1:3000	60	61.0	+1:3000
1:3500	27.0	25.0	+1:3500	60	60.0	1:3500
F						
1:3000	27.0	24.0	+1:3000	60	71.0	+1:3000
1:3500	27.0	26.0	+1:3500	60	66.0	+1:3500

HCl. Each cc. is equivalent to 0.10 mg. of N. F. Reference Pepsin.

Apparatus

(1) *Constant Temperature Bath.*—Water bath thermostatically controlled to maintain a temperature of 52° ± 1°.

(2) *Turbidimeter.*—A photoelectric instrument capable of making turbidimetric readings. In this work we have used a Pfaltz and Bauer Fluorophotometer Model B using a light source of 440 mμ.

(3) *20-Cc. Matched Pyrex Test Tubes.*—Clean test tubes with acid cleaning mixture and dry thoroughly.

Procedure

Transfer exactly 5 cc. of the albumen substrate to each of three test tubes marked B, B_1, and B_2. To one of three matched 20-cc. Pyrex test tubes marked A add exactly 5 cc. of 0.3% HCl solution of Pepsin containing 0.10 mg. of Pepsin 1:3000 per cc. To the second matched Pyrex test tube marked A_1 add exactly 4.30 cc. of a 0.3% HCl solution of Pepsin containing 0.10 mg. of Pepsin 1:3000 per cc. and 0.70 cc. of 0.3% HCl. To the third matched Pyrex test tube marked A_2 add exactly 5 cc. of Reagent N. F. Reference Pepsin (Solution B). Pour the contents of tube B into tube A, the mixture into tube B, and back to tube A. Repeat this mixing process with each pair of tubes.

When mixing is complete immerse the tubes in a water bath so that the level of the contents of the tubes is below that of the water. Maintain a temperature of 52° for forty-five minutes. At the end of this period quickly remove the tubes and place them in an ice water bath for at least five minutes.

Place the tube containing the N. F. Reference Standard in a Pfaltz and Bauer Fluorophotometer and set the galvanometer reading at 60 using a light source of 440 mμ. The tube containing 5 cc. of the solution of Pepsin being assayed should read not less than 60 and the tube containing 4.30 cc. of the solution of pepsin being assayed should not read more than 60 on the galvanometer.

Table III shows a comparison of the present method and the proposed method.

In the event the sample being assayed is stronger or weaker than the Reference Standard a true evaluation can be obtained by using a smaller or larger aliquot of the sample and repeating the assay.

CONCLUSION

A rapid method for standardizing pepsin involving a modified substrate and procedure is presented in which accuracy is improved and closer evaluation is possible.

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Studies on the Camphor Basil, *Ocimum Kilimandscharicum* Gürke*

By HEBER W. YOUNGKEN and WILLIAM E. HASSAN, Jr.

Detailed descriptions of the histology of the stem, leaf, bracts, and flowering parts of the Camphor Basil are presented. The yield of camphor is also recorded.

THE PUBLISHED report by Lowman and Kelly in 1945 (2) disclosing the results of their investigations of the Camphor Basil which they grew successfully on an experimental scale at Arlington Farms, Va., and at Beltsville, Md., and from which they isolated a camphor in purified form having physical and chemical properties similar to natural camphor (from *Cinnamomum Camphora*) incited the interest of the authors whose objectives were to determine the habit, the anatomical characteristics, and chemical properties of *Ocimum kilimandscharicum* Gürke as cultivated in New England (Fig. 1).

Fig. 1.—Camphor Basil, *Ocimum kilimandscharicum* Gürke, × 1/3.

Seeds of the Camphor Basil were generously supplied by Dr. Lowman of the U. S. Department of Agriculture Horticultural Station at Beltsville, Md. These seeds were planted in the greenhouse of the Arnold Arboretum at Jamaica Plain, Boston, Mass. The seedlings were then transplanted, in early May, to the Medicinal Plant Garden of the Massachusetts College of Pharmacy, where they were grown in moderately limed soil having a *p*H of 5.57. Crops were planted and harvested in 1946 and 1947. Not a plant was lost in the garden, all persisting in a healthy condition through the flowering and fruiting stages. Observations were made on these plants during the flowering and fruiting periods, studied in the College laboratories, and some new facts ascertained regarding their morphology. The general taxonomic characteristics of these plants agreed with the original description of the species found in the Kenya Colony by Gürke (3), except that they were annual in habit.

The purpose of this paper is to report the results of our investigation on the growth habit and structure of *Ocimum kilimandscharicum* cultivated in Boston, as well as the results of preliminary studies made on the oil and camphor distilled from the aerial portion of this cultivated species gathered on October 25, 1947.

GROWTH HABIT AND GROSS MORPHOLOGY

While the Camphor Basil, as described by Gürke (3), is a shrubby plant in its East African habitat, it is an annual in the latitude of Boston where the plants possessed the general characteristics of grayish green, bushy herbs attaining a height of from 1 1/2 to 2 1/2 ft. From a woody fibrous root system arises a brownish, hairy, subquadrangular stem with many branches beginning near the base. The branches are villose with white hairs and possess numerous petiolate, pubescent to villose leaves and terminal, pubescent, spicate racemes of verticillasters.

The leaves are ovate to ovate-lanceolate to broadly elliptic and measure up to 6.6 cm. in length, the petioles up to 1.5 cm. long, and the lamina from 1.0 to 2.7 cm. wide. These leaves are dull green in color and exhibit an acute summit, a cuneate base, a crenate-serrate to serrate margin in the upper portion of the leaves, and an upper and lower surface

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Presented to the Pharmacy Subsection of the Medical Sciences Section of the American Association for the Advancement of Science, Chicago meeting, 1948.

which are appressed, hairy, and glandular-punctate. Upon crushing or bruising the leaves, a strong camphor odor is emitted.

The flowers occur in 4- to 6-flowered whorls, each whorl subtended by 2 small bracts on long villose racemes, the latter up to 28 cm. in length. Each flower consists of a greenish purple, campanulate, bi-labiate calyx up to 4 mm. in length whose upper lip is orbicular with a mucronate apex and the lower lip 4-toothed, the two lower teeth subulate, longer and narrower than the lateral, broadly ovate to ovate-acuminate teeth.

The corolla is tubular-bilabiate and measures from 7 to 10 mm. in length, the upper lip weakly rose-red with white, ciliate, hairy margins, the lower lip white and ciliate-hairy on the lower face.

The 4 didynamous stamens possess long filaments, the upper filaments with a downward curved, hairy appendage near the base. The anthers are versatile. The style is long, exserted, and terminates in 2 digit-like style arms.

The nutlet fruits are ovoid to ovoid-oblong, rounded at one end, truncate to narrowly rounded at the other, and exhibit an elongated, triangular, flattened area on one side. These nutlets possess a black to brown testa and measure up to 1.6 mm. in length.

Two bracts subtend each verticillaster. They are short-petiolate, ovate, with cuspidate apex, entire margin and rounded tapering base. Their surfaces and margins are villose and glandular hairy. Those measured on one raceme were up to 10 mm. long and 5 mm. wide.

HISTOLOGY OF THE STEM

Transverse sections of the stem (Fig. 2) of *Ocimum kilimandscharicum* are subquadrangular in outline and present the following characteristics:

(*a*) Epidermis of more or less rounded cells covered by a prominent cuticle and bearing nonglandular and glandular hairs. The nonglandular hairs are of two types, *viz.*, short, unicellular, conical hairs which arise from 1, 2, or 3 epidermal cells and uniseriate, papillose hairs with a usually short, broad, basal cell and pointed end cell. The latter type is usually composed of 5 to 6 cells but occasionally 7- to 8-celled hairs have been found. The glandular hairs possess a one-celled stalk and a head of 1, 2, 4, or 8 secretory cells.

(*b*) Cortex of about 5 layers of cells, except in the rib zones where it is broader. Just beneath the epidermis, collenchyma occurs which constitutes a broad zone in the rib regions and a narrow zone in the intercostal regions. Beneath this occur tangentially elongated, ovoid, cortical parenchyma cells.

(*c*) Endodermis, contrary to Blanque and Maheu (1) who are stated to have wrongly identified this species as *Ocimum canum*, we have found endodermis differentiated in the younger portion of the stem into a prominent layer of clear, more or less radially elongated cells. In older portions of the stem, however, this differentiation is lost and the endodermal cells appear tangentially elongated.

(*d*) Pericycle, a zone of parenchyma in which are imbedded oval to irregularly oblong groups of pericyclic fibers forming an interrupted circle. These groups are largest in the rib regions. The fibers possess a thin wall and a broad lumen. They are nonlignified in young parts of the stem, becoming lignified in the older, thicker portions.

(*e*) Open collateral fibrovascular bundles consisting of a narrow phloem of sieve tissue devoid of fibers. A prominent cambium is present and a relatively broad xylem of narrow wood wedges and wood rays. The wood wedges are composed of lignified wood fibers and tracheae, the latter arranged in radiate fashion and most numerous in the early wood.

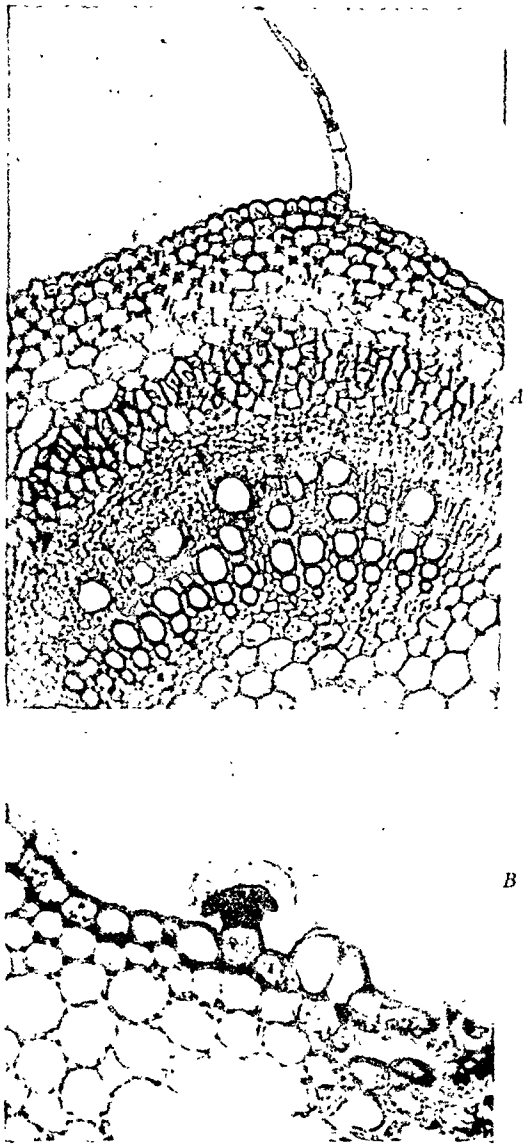

Fig. 2.—*A*, transverse section of the stem of *Ocimum kilimandscharicum*, × 100. *B*, transverse section of outer portion of the same stem showing a glandular hair, × 200.

(*f*) Pith, a broad central zone of pitted parenchyma.

In longitudinal sections, the tracheae and the tracheids exhibit spiral and pitted markings.

HISTOLOGY OF THE LEAF BLADE

This leaf (Fig. 3) has a midrib which is concavo-convex in cross section and exhibits an oval to crescentic meristele of bundles differentiated into phloem and xylem regions. The phloem is devoid of phloem fibers. The xylem possesses narrow wood wedges, each consisting of radially arranged single rows of tracheae separated by wood fibers, both with lignified walls. Surrounding the bundle zone is round parenchyma devoid of calcium oxalate and starch. Above, the palisade parenchyma encroaches slightly laterally into the midrib zone. The epidermis is composed of rounded to tangentially elongated cells from which arise uniseriate, nonglandular hairs. The midrib is reinforced by a layer of collenchyma beneath the epidermis.

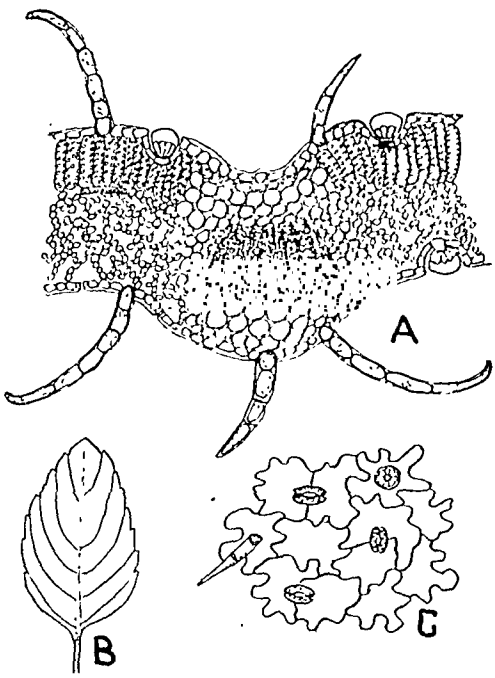

Fig. 3.—Leaf of *Ocimum kilimandscharicum*. *A*, transverse section through the midrib and the interneutral regions. *B*, entire leaf. *C*, surface section of the lower epidermis.

The interneural region of the lamina exhibits a layer of epidermal cells on both surfaces which are somewhat rounded to slightly tangentially elongated in cross section and deeply sinuate wavy in surface view. The cells of the lower epidermis are smaller than those of the upper epidermis. Ovate to elliptical stomata occur, in both epidermises, which measure up to 42 μ in length. They are of the Labiate type. A cuticle covers the epidermal cells. Both epidermises show numerous crypts from the base of each of which arise sessile or short-stalked hairs, the glandular heads of which exhibit from 4 to 8, occasionally 12, secretory cells and an upraised subglobular to balloon-shaped cuticle. Between the cells of the gland and the cuticle, oil and camphor are deposited. The cells lining the crypt are tangentially elongated in cross section. In addition, both epidermises possess numerous nonglandular hairs mostly of the uniseriate, papillose type. While most of the hairs found are composed of 5 to 6 cells, a few have been found up to 7 and 8 cells. The basal cell of each hair is usually broad and the distal cell, narrow and pointed. Some of the nonglandular hairs measured up to 1350 μ in length.

Beneath the upper epidermis there occurs a single layer of palisade parenchyma which occupies in different regions from one-third to one-half the mesophyll. The spongy parenchyma is composed of irregularly rounded to elliptic cells forming an open network of cells surrounding prominent air spaces. Just above the stomata of the lower epidermis, the spongy parenchyma cells surround large substomal air spaces. The vascular strands coursing through the spongy parenchyma exhibit tracheary elements with spiral thickenings.

TABLE I.—MOISTURE CONTENT[a]

Sample No.	Wt. of Sample, Gm.	Wt. of Sample After Drying	% Moisture
1	98.7	26.2	73.4
2	95.4	21.4	77.5
3	97.2	23.8	75.5
4	97.9	23.7	75.7
Av.	97.3	23.8	75.5

[a] The moisture content was determined by cutting the drug up into small pieces, placing in large evaporating dishes, and placing in a 37° constant temperature oven until the sample attained constant weight.

TABLE III.—PHYSICAL CHARACTERISTICS OF THE OIL

Appearance	Yellowish liquid
Odor	Camphoraceous
Specific gravity	0.9225 at 25°
Refractive index[a]	1.4752 at 20° 1.4735 at 25°

[a] The refractive index was determined according to the method described in the U. S. P. XIII (5).

TABLE IV.—PHYSICAL CHARACTERISTICS OF THE CAMPHORACEOUS DEPOSIT AND ITS DERIVATIVES

Appearance	White, crystalline, granular mass
Odor	Resembling that of camphor
Taste	Aromatic and pungent
Solubility	Insoluble in water 1 Gm. soluble in 1 cc. alcohol 1 Gm. soluble in 0.5 cc. chloroform Readily soluble in the extracted oil
Melting point	174 to 176°
M. p. of oxime	116°
M. p. of semicarbazone[a]	234°

[a] The derivatives were prepared according to the methods of Shriner and Fuson (4) and the melting points according to the U. S. P. XIII (6).

TABLE II.—CAMPHOR AND OIL CONTENT[a]

Sample No.	Wt., Gm.	Time of Distn., Hr.	Part Used	Camphor, %	Oil, %
1	70.0	3.5	Entire fresh herb	0.98	0.43
2	70.0	4.0	Entire fresh herb	0.83	0.36
3	50.0	4.0	Leaves and flowering tops dried at 37° for 48 hr.	2.15	2.20
4	50.0	4.0		2.12	2.00
5	50.0	4.0		2.91	2.80
6	50.0	4.0		3.01	3:00

Av. Camphor Content, dried leaves and flowering tops, 2.54%.
Av. Oil Content, 2.50%.

[a] The oil was collected by the U. S. P. method (7) for collecting oils lighter than water and was freed from any camphor in solution by freezing until no more camphor crystallized out.
The camphor was collected on the condenser of the above apparatus, removed by dissolving in chloroform, recrystallized, and dried over sulfuric acid for forty-eight hours.

HISTOLOGY OF THE FLORAL PARTS AND BRACTS

The calyx, corolla, and bracts possess epidermal lls with curvilinear vertical walls, stomata, and pillose, nonglandular hairs similar to those which cur on the leaves except that there are more of the and 8-celled, uniseriate type. These hairs are up 1460 μ in length. The glandular hairs show ostly 4 secretory cells, are stalked, and not sunken crypts in the corolla. The bract shows three rves, a ciliate margin, and its apex is pilose-hairy. The filaments of the stamens consist of (*a*) an idermis of elongated epidermal cells having thin alls and containing minute yellowish, globoid to oid bodies of protein, occurring in aggregates ithin the nuclei, and irregularly shaped chromoastids in their cytoplasm; (*b*) a narrow zone of ongated, thin-walled parenchyma; (*c*) a central iscular strand of sieve tissue and spiral tracheae. ist above the insertion point of the filaments, they e covered with numerous stout, papillose, unillular, uniseriate, nonglandular hairs, the latter onsisting of up to 3 cells, conspicuously jointed, the id cell either obtuse or pointed.

The pollen grains are subspheroidal with a reticute exine and measure up to 72 μ in diameter.

CONSTITUENTS

Lowman and Kelly (2) in their report gave figures or the yield of oil and camphor which had been exracted from material gathered at various times durig the summer. The figures stated below are for iaterial which was extracted from plants gathered ist before frost on October 25, 1947.

The physical constants of the camphor and oil and ata pertaining to the moisture, camphor, and oil ontent are recorded in Tables I–IV.

SUMMARY

1. Camphor Basil, *Ocimum kilimandscharicum*, possesses the growth habit of an annual herb i the latitude of Boston, Mass., where the pecies can be grown to maturity if planted in arly spring.

2. Plants of Camphor Basil grown in the Medicinal Plant Garden of the Massachusetts College of Pharmacy are described. These were ound to agree in their essential taxonomic characters with the description of the species by Gürke and Engler.

3. Detailed descriptions are given of the histology of the stem, leaf, bracts, and flowering parts, and new facts reported on their microscopic structure.

4. The chief diagnostic histological elements of the stem, leaf, and bract are as follows: (*a*) uniseriate, papillose, nonglandular hairs, usually of up to 5 or 6 cells but occasionally of 7 or 8 cells, each with a short, broad, basal cell and a narrow, pointed, distal cell; (*b*) short, conical, papillose, 1- to 2-celled hairs; (*c*) sessile to short-stalked glandular hairs with heads of 1, 2, 4, or 8 secretory cells and an upraised cuticle, many of which contain camphor and volatile oil; (*d*) numerous crypts occurring on the epidermis of the foliage leaves from the bases of which some of the glandular hairs arise; (*e*) deeply sinuate, vertical walls of both upper and lower epidermises of the leaf and bract.

5. The endodermis in transverse sections of the younger portion of the stem consists of large, clear, more or less radiately elongated cells. In older portions of the stem this differentiation is lost, the endodermal cells there appearing tangentially elongated.

6. A camphor and oil are present in *Ocimum kilimandscharicum* each of which possesses physical characteristics similar to the natural camphor and oil obtained from *Cinnamomum Camphora*.

7. The dried leaves and flowering tops of the Camphor Basil grown in Boston and harvested in late October yielded an average of 2.5% of oil and 2.54% camphor.

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(3) Gürke in Engler, A., "Die Pflanzenwelt Ost Africa," Theil, A., C. D. Riemer, Berlin, 1895, pp. 349–50.
(4) Shriner, R. L., and Fuson, R. C., "Indentification of Organic Compounds," John Wiley & Sons, New York, 1935, p. 145.
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(6) *Ibid.*, p. 667.
(7) *Ibid.*, p. 715.

Contribution to the Chemistry and Pharmacology of the *Digitalis purpurea* Glycosides*

By E. W. McCHESNEY, F. C. NACHOD, M. E. AUERBACH, and F. O. LAQUER

Pure samples of gitoxin, digitoxin, and their aglucones have been prepared. An analytical method has been devised for the determination of the proportion of digitoxin and gitoxin plus gitalin in a mixture. The principle involved depends upon the colors developed by the action of concentrated sulfuric acid on digitoxigenin and gitoxigenin.

IN RECENT years there has been a considerable increase of interest in the cardiac glycosides obtained from *Digitalis purpurea* (foxglove), due chiefly to the work of Cloetta, Windaus, and Jacobs, and their co-workers. Purified glycoside preparations, usually designated as digitoxin, have been available commercially in Europe for many years, and more recently in this country. The digitoxin content of these preparations probably exceeds 90 per cent but, unless especially purified, some of another active *D. purpurea* glycoside, namely gitoxin, is also present.

The pharmacology of gitoxin has never been completely worked out, presumably because sufficient quantities of authentic material have not been available to those interested. The pharmacology of digitoxin has, of course, been quite thoroughly studied. Fromherz and Welsch (1) compared the toxicity of the two substances in frogs and cats. In the former species they found gitoxin to have an activity equivalent to 40 per cent of that of crystalline digitoxin, and in cats, using the Hatcher-dose method (2), they found an activity equivalent to 70 per cent of digitoxin. Cloetta (3*b*) gave the frog and cat toxicity of his "bigitalinum" (apparently identical with gitoxin) as about 35 per cent of that of digitoxin. Chen, Chen, and Anderson (4) reported determinations of the toxicity of a number of cardiac glycosides but found gitoxin too insoluble in alcohol and water to permit a determination of its toxicity. We have not encountered this difficulty.

The reaction of gitoxin with sulfuric acid to produce a brilliant red color is well known, having been observed by Cloetta (3*b*) and others. We felt that it might be possible to develop this qualitative reaction into a quantitative method for the analysis of a mixture of the two glycosides, since with sulfuric acid, pure digitoxin gives only a yellowish brown color. Such a method would be particularly useful in the process of purification of both substances, and for the analysis of commercial preparations which may be mixtures of the two.

* Received December 19, 1947, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

DESCRIPTION OF PREPARATIONS USED

Digitoxin.—U. S. P. quality digitoxin was recrystallized several times from 75% methanol, using charcoal for complete decolorization. The crystallizations were repeated until a constant melting point and crystal form were obtained. There is no single definitely established criterion in the literature by which a pure preparation of digitoxin can be identified, but the melting point of 255–257° given by Windaus and Freese (5) seems to be the one most commonly obtained.[1] This, however, depends on the rate of heating, and an impure preparation may give nearly as high a value. In all cases described below the melting point was taken with a Kofler hot stage microscope, using an average temperature rise of about 15°/min., or, alternatively, by heating a bath to about 20° below the expected melting point before immersing the melting-point tube, then increasing the temperature at the rate of 5°/min. All readings recorded are corrected for stem exposure.

Preparation A.—Opaque colorless needles, m. p. (hot stage) 253–255°, alternate method, 238–240°. Chloroform solubility, 1:30 by weight. Elementa- analysis: C, 64.35%; H, 8.23%. Calculate C, 64.38%; H, 8.41%. $[\alpha]^{23}_{D} = +15 \pm 1°$ (1 in 3:1 chloroform-methanol); in literature $[\alpha]^{21}_{D}$ +21° (0.55% in $CHCl_3$) (6). The chlorofor solubility (1:92) of the preparation on which t latter value was recorded leaves its authentici somewhat in doubt.

Preparation B.—Opaque colorless plates ar needles. M. p. (hot stage), 253°, alternate metho 236–237°. Chloroform solubility, 1:20. Eleme tary analysis: C, 64.07%; H, 8.58%. This prep ration is evidently not quite pure in spite of its e cellent physical characteristics.

Gitoxin.—This substance was prepared fro commercial chloroform-insoluble residues by tl method of Windaus and Schwarte (7). The crysta line material obtained consisted of oblong rectangu lar plates or prisms resembling closely those picture by Stoll (8). M. p. (hot stage), 278°; in literatu (3*b*), 282°; alternate method, 263–265°; in litera ture (9), 266–269°. Chloroform solubility, 1:11,00 by weight (determined colorimetrically on an aliquo of a saturated solution by the method of James Laquer, and McIntyre (10)). Elementary analysis

[1] When their preparation was crystallized from aqueou alcohol, it melted at 233–235°.

[2] We attempted to solve the problem of the low solubilit of gitoxin by dissolving it in $CH_3OH:HCOOH$ = 1:4 This gave $[\alpha]^{22}_{D} = +70°$, and for digitoxin A $[\alpha]^{22}_{D} = +54°$ The rotation of gitoxigenin was not enhanced by this solven mixture.

C, 62.94%; H, 8.11%; Calculated: C, 63.05%; H, 8.26%. $[\alpha]_D^{24} = +5 \pm 1°$ (1% in pyridine).[2] In literature $[\alpha]_{Hg}^{20} = +3.5°$ (in pyridine) (11).

Digitoxigenin.—This substance was prepared from pure digitoxin as described by Cloetta (3a). M. p. by either method, 247–250°; in literature, 251° (13). Analysis: C, 73.86%; H, 9.02%; Calculated: C, 73.76%; H, 9.15%. $[\alpha]_D^{24} = +20 \pm 1°$ (1% in 3:1 chloroform-methanol). In literature, $[\alpha]_D^{17} = +19.1°$ (12).

Gitoxigenin.—Prepared from pure gitoxin according to Windaus and Schwarte (7). M. p. by either method, 222–224°; in literature, 224–225°. Analysis: C, 70.90%; H, 8.72%; Calculated: C, 70.73%; H, 8.74%. $[\alpha]_D^{23} = +35 \pm 1°$ (1% in 3:1 chloroform-methanol). In literature, $[\alpha]_D^{20} = +33.6°$ (in methanol) and $[\alpha]_{Hg}^{20} = +38.5°$ (in methanol) (11).

EXPERIMENTAL

Color Reaction of Gitoxigenin and Digitoxigenin with Sulfuric Acid.—Windaus and Schwarte noted that gitoxigenin gives a red color with ferric chloride in the presence of strong sulfuric acid. For example, if 50 γ of the aglucone are dissolved in 5 cc. of the concentrated acid and 1 drop of 5% ferric chloride solution is added, a beautiful cherry-red color develops within a few seconds. This color reaction does not seem well adapted to colorimetric work since the intensity increases rapidly up to about 5 minutes, and then the red color fades and is replaced by brown. On the other hand, if the ferric chloride is not added, the same color develops slowly, reaches a maximum in about twenty-four hours, and, to the naked eye, remains unchanged for several days. The time factor is thus not critical. Digitoxigenin treated similarly gives at first a pale lemon-yellow color which gradually deepens. The glycosides give similar color reactions, but the aglucones are much better adapted to colorimetric work than are the glycosides since with the latter there is admixed a brown color resulting from the reaction of sulfuric acid on the digitoxose. The absorption spectra of the aglucones in sulfuric acid were studied in a Beckman Spectrophotometer and are shown in Fig. 1.

It may be seen from the figure that the different behavior of the two aglucones provides a basis for the analysis of a mixture. At about 420 mμ there is an isosbestic point, while at 525 mμ the absorption coefficient of gitoxigenin is about 10 times that of digitoxigenin. (In a photoelectric colorimeter, however, using a 520 filter, the ratio of optical densities is not so favorable, being rather of the order of 5–7 times.) The curves show, furthermore, that after twenty-four hours no rapid change is taking place in the gitoxigenin color; therefore approximately this interval was used in the colorimetric work.

It is of interest to note at this point that metallic catalysts other than iron may be used for more rapid color development. If a drop of dilute copper sulfate is added, the gitoxigenin solutions develop a maximum color in about four hours and fade somewhat after twenty-four hours. The red color is about 50% more intense at the maximum than is ever developed in the absence of copper, while with digitoxigenin the color intensity is essentially unchanged by the addition of copper. However, the use of this technique has given results which are no more reliable than those obtained without copper, and the idea has not been pursued further. Vanadic and mercuric salts also serve excellently as catalysts for this color reaction, suggesting that it is oxidative in nature.

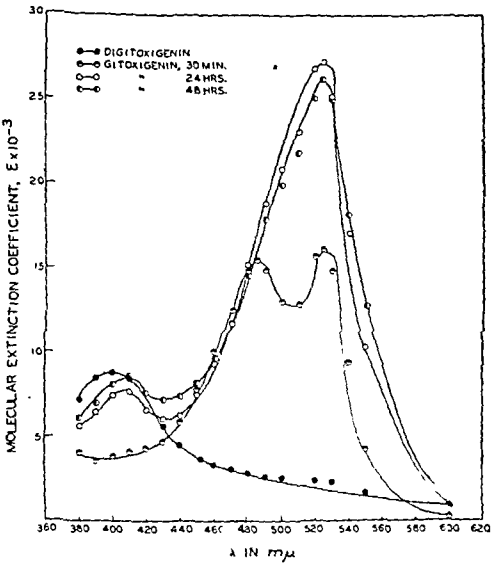

Figure 1.

ANALYTICAL PROCEDURE

Preparation of Samples and Standards.—Twenty milligrams of the sample are hydrolyzed by refluxing for forty-five minutes with 10 cc. of 0.13 *N* HCl in 50% ethanol, as directed by Windaus and Schwarte. The hydrolyzate is transferred to a separatory funnel with the aid of a little alcohol, and three volumes of water are added, followed by enough *N* NaOH to neutralize the HCl. The aglucone is extracted by shaking twice with 25 cc. chloroform, and the chloroform solution is made up to 100 cc. with alcohol. This solution contains almost exactly 100 γ of aglucone per cc., but the quantity is not critical except in the standards used for calibration.

Portions of 1 cc. of the aglucone solution are evaporated to dryness in scrupulously clean test tubes, and 5 cc. of concentrated sulfuric acid are added. Extreme accuracy in measurement is not necessary. The tubes are allowed to stand in a dry place (in bell jar or desiccator) for twenty to twenty-two hours. The color is then read in a photoelectric colorimeter against a sulfuric acid blank, using both 420 and 520 filters. An instrument which gives readings directly proportional to the optical density is preferable. The 520 reading is divided by the 420 reading and the ratio recorded. If a series is properly set up all of the 420 readings will be identical within ±5%. Any reading which is very high suggests contamination with organic matter and should be repeated.

Standards consist of hydrolyzed pure gitoxin and digitoxin treated as previously described.[3,4] A series for calibration is most conveniently set up as follows: In a series of 6 test tubes labeled 100, 80, 60, 40, 20, and 0 measure the following quantities of hydrolyzed digitoxin, respectively: 1.0, 0.8, 0.6, 0.4, 0.2 0. cc. Then measure in the following quantities of hydrolyzed gitoxin, respectively: 0, 0.2, 0.4, 0.6, 0.8, 1.0 cc. Each tube then contains a total of almost exactly 100 γ of aglucone, with the percentage of digitoxigenin ranging from 100 to 0 in steps of 20%, and gitoxigenin making up the balance. The solvents are evaporated off and the procedure for color development outlined above is followed.

About five repetitions of this series will give a calibration curve[5] which is a straight line, as shown in Fig. 2. The individual points plotted represent a sixth series of tubes, and their standard deviation from the established curves is about ±2%. Unknown materials have been plotted against this calibration curve with typical results as given in Table I. When known mixtures have been hydrolyzed (samples 1–3) they have given the expected results, and experiments involving the addition of pure standards to materials of unknown composition have been successful (samples 9, 11–13). Since the results are obtained as ratios, they must be multiplied by the total glycoside content (10) to give the absolute values for the individual glycosides.

It is of interest to record here that, although digitoxin and gitoxin give equal color values by the James-Laquer-McIntyre method, crystalline gitoxin gives only 28% of the color value of digitoxin (Preparation A) by the Anderson-Chen method (13). Similarly, by the latter procedure, digitoxigenin gives 195%, and gitoxigenin 37%, as much color as digitoxin (A). The color reaction has been attributed to the active methylene hydrogen of the lactone ring (14), but for some reason this atom is less active in gitoxigenin and gitoxin. The difference is not solely one of the reaction time since the color intensity with gitoxin never equals that of digitoxin. It has also been noted (15) that gitoxin is less active when the analysis is made by the Bell-Krantz method (16).

It must be concluded, therefore, that the James *et al.* method is a more accurate index of total glycoside content than the Anderson-Chen, while the latter method is more nearly specific for digitoxin

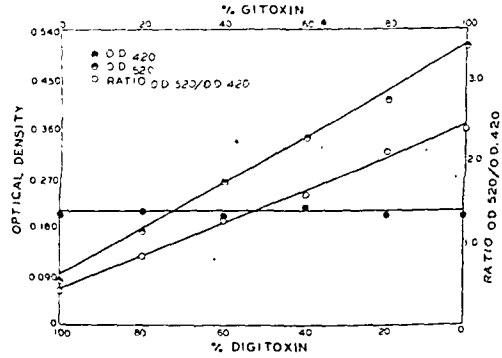

Figure 2.

TABLE I.—ANALYSIS OF SOME TYPICAL GLYCOSIDE PREPARATIONS FOR GITOXIN/DIGITOXIN DISTRIBUT

Sample	Composition	No. of Trials	$\frac{D_{520}}{D_{420}}$, Av.	% Gitoxin[a] ± S. E.	% Gitoxi Calculat
1	5 mg. Digitoxin (A) + 15 mg. Gitoxin	4	1.91	72.8 ± 1.3	75
2	10 mg. Digitoxin (A) + 10 mg. Gitoxin	9	1.46	50.2 ± 3.7	50
3	15 mg. Digitoxin (A) + 5 mg. Gitoxin	5	0.93	24.2 ± 1.6	25
4	20 mg. Digitoxin (B)	5	0.49	2.2 ± 0.9	...
5	U. S. P. Digitoxin Reference Standard	6	0.51	3.2 ± 1.4	...
6	Commercial U. S. P. XIII Digitoxin	5	0.67	10.6 ± 0.5	...
7	Commercial U. S. P. XIII Digitoxin	5	0.43	−0.6 ± 0.8	...
8	Crude chloroform insoluble residues	6	1.70	62.0 ± 1.8	...
9	10 mg. Sample 8 + 10 mg. Digitoxin Std. hydrolyzed together	6	1.08	31.0 ± 0.96	31[b]
10	Partially purified Gitoxin	7	2.12	83.5 ± 2.2	...
11	0.5 cc. from Sample 8 + 0.5 cc. Digitoxin Std.	5	1.08	31.0 ± 1.7	31[b]
12	0.5 cc. Sample 11 + 0.5 cc. Digitoxin Std.	5	1.30	42.5 ± 0.9	41.8[b]
13	0.5 cc. Sample 6 + 0.5 cc. Gitoxin Std.	5	1.57	55.2 ± 2.3	55.3[b]

[a] And/or *Gitalinum cristallisatum*. Digitoxin makes up the remainder of 100%.
[b] Calculated assuming that the values already obtained for the unknown portions of these samples are correct.

[3] Samples of digitoxin and gitoxin may be obtained from the Special Chemicals Division of Winthrop-Stearns, Inc., 1450 Broadway., New York, N. Y.

[4] If pure aglucones are used as standards the calibration curve has a somewhat steeper slope, chiefly because the readings at 420 mμ are low and inconsistent. This could have two possible interpretations: (1) the digitoxin and gitoxin preparations used as standards are not entirely pure, or (2) a trace of impurity in
development. The second
since the calibration curve
samples of standards gives
the preparations. For example, against the aglucone standards, the U. S. P. reference standard would analyze 8% gitoxin and all the other results would be shifted toward the 50% mark.

[5] Different samples of sulfuric acid may give different calibration curves depending on their content of heavy metals.

than the former. For example, a preparation co
taining 70% digitoxin, 30% gitoxin would ass
about as follows (in terms of digitoxin): James *et*
method, 100%; Anderson-Chen method, 78%; c
method, 96%. (NOTE: it is assumed that the c
potency of gitoxin is 90% of that of digitoxin; s
below.)

Interfering Substances.—Other constituents
D. purpurea such as pigments, tannins, chlorophy
lipids, etc., are usually well eliminated by the time
preparation has reached a total glycoside conte
of 95% or more, which probably should be the ca
before the present method is applied. The saponi

(digitonin, gitonin, and tigonin) might still be present in minute amounts.[6] These substances are difficult to hydrolyze under the conditions prescribed for the glycosides, and they yield different genins. Gitogenin, sometimes a contaminant, gives a pale yellow color with sulfuric acid, but of a lesser intensity than that of digitoxigenin (19). It is unlikely, therefore, that these would offer any serious interference. If, however, the substance described by Cloetta (3*b*) under the name *Gitalinum cristallisatum* is actually present in the leaves of *D. purpurea*, it would affect the results. It is believed to be a glycoside consisting of 2 molecules of digitoxose and a monohydrate of gitoxigenin. The per cent of digitoxin in a given preparation would, therefore, be as determined from the graph, but what is designated as "per cent gitoxin" would presumably represent gitoxin plus *Gitalinum*. Some entirely different principle would have to be applied to the analysis of a mixture of the latter two substances if their color reactions with sulfuric acid are actually the same.

Ultraviolet Absorption.—As would be expected, the glycosides show chiefly end-absorption. However, when examined in ethanol solution over the range 212–265 mμ, gitoxin shows a definite maximum at 219 mμ with $E^{1\%}_{1\,cm.} = 191$, while digitoxin shows no definite maximum but at 215 mμ, $E^{1\%}_{1\,cm.} = 263$.

Toxicity of Gitoxin.—The crystalline glycoside was ground to a fine powder and 25 mg. were dissolved in 73 cc. of 95 % ethanol by warming to 50° and shaking occasionally until solution was complete. Water was carefully added to a volume of 100 cc.; the solution remained clear. This was then diluted in the ratio 4/35 with 0.9% NaCl in 20% ethanol, again without precipitation. When this solution was administered to cats the average lethal dose was 15.5 ± 0.57 cc. or 0.443 ± 0.014 mg. of gitoxin. The U. S. P. Digitoxin Reference Standard, tested concomitantly, gave an average lethal dose of 0.375 ± 0.015 mg.; therefore, gitoxin would appear to be 83% as toxic as digitoxin. LaWall and Harrisson, Consultants, of Philadelphia, tested the same sample of gitoxin and reported a toxicity equivalent to 1859 U. S. P. Digitalis Units per Gm., or about 95% of the 1900–2000 units found for the average U. S. P. XIII digitoxin.

DISCUSSION

Gitoxin has been found in this work to have a biological activity closely approaching that of digitoxin. This is logical since the view is generally accepted that the lactone ring is responsible for the toxicity (17). There would prove to be little justification for an expensive process of purification to remove this "impurity," in the event that the pharmacological and therapeutic properties of digitoxin and gitoxin are otherwise similar.

The analytical procedure described in this paper gives an indication of the relative amounts of digitoxin and gitoxin (and/or *Gitalinum*) present in the simple case where the preparation consists almost entirely of glycosides. It might conceivably have some application to the analysis of the lanatosides (Digilanid B) since digoxigenin, as well as digitoxigenin, does not give a red color with sulfuric acid (18).

Purified glycosides which meet present U. S. P. requirements for digitoxin may contain an appreciable amount of gitoxin (and/or *Gitalinum*). The criterion of chloroform solubility prescribed in U. S. P. XIII does not guarantee the absence of gitoxin.

The authors gratefully acknowledge the data contributed to this paper by two members of the staff of Winthrop-Stearns, Inc. Dr. H. B. Corbitt conducted the analyses by the Anderson-Chen method, and Mr. K. D. Sprague determined the lethal dose of gitoxin for cats.

SUMMARY

1. Analytically pure samples of gitoxin, digitoxin, and their aglucones have been prepared.

2. Gitoxin has been found to have a toxicity in cats only slightly less than that of digitoxin.

3. Digitoxigenin and gitoxigenin may be distinguished by means of the colors they develop in concentrated sulfuric acid. This reaction has been developed into an analytical method which gives the proportion of digitoxin and gitoxin plus gitalin in a mixture. This method should be of value in following purification processes and in the analysis of commercial preparations.

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[6] U. S. P. XIII specifications eliminate these substances qualitatively.

Effect of Particle Size, Type of Base, and a Wetting Agent on Three Antiseptic Ointments*

By LEE H. MACDONALD and R. E. HIMELICK†

In the official ointments of ammoniated mercury, yellow mercuric oxide, and calomel, a reduction in the particle size of the mercurial usually contributes to increased antiseptic activity, as measured by the agar plate method. Likewise, the addition of the wetting agent dodecylamine hydrochloride to the ointments causes a moderate increase in the zones of inhibition. Substituting an emulsion base (containing cetyl alcohol, Carbowax, sodium lauryl sulfate, and water) for the official bases greatly increases the inhibition zones in all cases.

MANY STUDIES have been made to determine the relative effectiveness of grease-type and emulsion-type bases for antiseptic ointments. Gershenfeld and Brillhart (1), for example, found that ointments of ammoniated mercury, phenol, and mercuric chloride produced greater bacteriostatic action when the medicaments were incorporated into water-miscible bases. Fiero and Loomis (2) have reported the antiseptic activity of several official ointments to be enhanced by substituting emulsified bases for the official bases.

In the field of sulfonamide ointments, emulsion bases have been particularly recommended. Howard (3) determined that emulsion bases yielded high levels of sulfonamides to a surrounding aqueous medium, while grease and cold cream bases yielded little. Bases containing at least 50 per cent water were found by Aldrich and DeKay (4) to give optimum activity in sulfonamide ointments. They also observed increased penetrative power when surface-active agents were added.

The effect of the particle size of the active ingredients in antiseptic ointments has also been studied. Vichers, Snyder, and Gathercoal (5) found that a calomel ointment prepared from a specially processed colloidal calomel showed a much higher antiseptic power than one made from U. S. P. calomel, and they postulated that the decreased particle size might account for its increased effectiveness. Other explanations for this phenomenon have been offered, however (6). Fleming and Wolf (7) made a series of calomel ointments, varying the particle size of the calomel, and tested them as prophylactic agents for experimental syphilis in rabbits. They found an ointment made with 100 micron calomel to be relatively ineffective, with effectiveness increasing as particle size was reduced. Laug, *et al.* (8) also observed that reducing the particle size of calomel increased the penetration of mercury. They found that surface-active agents increased skin penetration when incorporated into an animal fat or emulsion base, but detected no effect when the agent was incorporated into a mineral base.

In this study we have attempted to evaluate the effect of (*a*) type of base, (*b*) particle size, and (*c*) the surface-active agent dodecylamine hydrochloride on the antiseptic activity of three commonly used mercury-containing ointments: ammoniated mercury, calomel, and yellow mercuric oxide.

EXPERIMENTAL

A series of ammoniated mercury ointments was prepared and tested for antiseptic activity by the agar plate method.[1] Formulas and zones of inhibition are indicated in Table I.

Results obtained with a series of yellow mercu oxide ointments are indicated in Table II.

Two series of calomel ointments were prepa and tested, one at the N. F. strength of 30% a another at the 10% level. The latter concentrat

TABLE I.—AMMONIATED MERCURY OINTMENTS

Ammoniated Mercury, %	Av. Particle Size in μ	Base	Dodecylamine Hydrochloride, %	Zon of Inhit tion Mm
5	8	U. S. P. XIII	0	5
5	8	U. S. P. XIII	0.1	6
5	8	Emulsion[a]	0	9
5	3	U. S. P. XIII	0	5
5	3	U. S. P. XIII	0.1	6
5	3	Emulsion	0	9
0	.	U. S. P. XIII	0	0
0	.	U. S. P. XIII	0.1	0
0	.	Emulsion	0	0

[a] The emulsion base used throughout this study contain 25% cetyl alcohol, 25% Carbowax 1500, 1% sodium lau sulfate, and 49% water.

[1] A 20-cc. portion of meat infusion agar is melted and cool to 45°. To this is added 0.2 cc. of an eighteen-hour bro culture of *Staphylococcus aureus* 209 P. This inoculat agar is then poured into a sterile Petri plate and allowed harden, after which 0.5 Gm. of the ointment is placed in t center of the agar. The plates are incubated at 37°, a read after forty-eight hours. Inhibition zones are measur from the edge of the ointment to the edge of the zone of co plete inhibition.

* Received Dec. 10, 1947, from the Research Division of The Upjohn Company, Kalamazoo, Mich.

† We wish to acknowledge the assistance of the Bacteriology Research Department of The Upjohn Company in compiling the bacteriological data for this study.

was chosen so that comparisons might be made with the colloidal calomel of Vichers, *et al.* (5), which does not lend itself well to incorporation into a 30% ointment. Formulas and zones of inhibition obtained with both series are shown in Table III.

TABLE II.—YELLOW MERCURIC OXIDE OINTMENTS

Mercuric Oxide %	Av. Particle Size in μ	Base	Dodecylamine Hydrochloride, %	Zone of Inhibition, Mm.
1	10	U. S. P. XIII	0	1
1	10	U. S. P. XIII	0.1	6
1	10	Emulsion	0	10
1	1.5	U. S. P. XIII	0	4
1	1.5	U. S. P. XIII	0.1	7
1	1.5	Emulsion	0	10
0	..	U. S. P. XIII	0	0
0	..	U. S. P. XIII	0.1	0
0	..	Emulsion	0	0

antiseptic efficacy of the grease-base ointments, as measured by the plate method.

SUMMARY

A series of ammoniated mercury, yellow mercuric oxide, and calomel ointments has been prepared and tested for antiseptic activity by the agar plate method. The effect of particle size, type of base, and the addition of a wetting agent on the size of the inhibition zone has been studied.

Reduction of particle size and the addition of the surface-active agent dodecylamine hydrochloride usually contributed to the production of an increased zone of inhibition in the grease-base ointments.

Substitution of an emulsion base for the grease base resulted in an increased inhibition zone in all cases.

TABLE III.—CALOMEL OINTMENTS

Calomel, %	Av. Particle Size in μ	Base	Dodecylamine Hydrochloride, %	Zone of Inhibition, Mm.
30	5	N. F. VIII	0	1.5
30	5	N. F. VIII	0.1	1.5
30	5	Emulsion	0	3.5
30	2	N. F. VIII	0	2
30	2	N. F. VIII	0.1	3
30	2	Emulsion	0	5
10	5	N. F. VIII	0	0
10	5	N. F. VIII	0.1	0
10	5	Emulsion	0	3
10	2	N. F. VIII	0	0
10	2	N. F. VIII	0.1	1.5
10	2	Emulsion	0	5
10	Colloidal[a]	N. F. VIII	0	7.5
10	Colloidal	N. F. VIII	0.1	8.5
10	Colloidal	Emulsion	0	10
0	....	N. F. VIII	0	0
0	....	N. F. VIII	0.1	0
0	....	Emulsion	0	0

[a] Less than 1 μ.

DISCUSSION

From these results it appears that ointments of ammoniated mercury, yellow mercuric oxide, and calomel are least effective in their present official bases, if the agar plate test can be taken as a criterion of their efficacy. In all cases the use of an emulsion-type base gave ointments showing greater zones of inhibition.

In most instances reduction in particle size of the mercury compound and the addition of 0.1% dodecylamine hydrochloride each contributed to the

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Stability Study of Penicillin Ointment*

By S. H. CULTER†

A number of penicillin ointments using various types of bases with sodium, calcium, and potassium salts of penicillin of varying degrees of potency were prepared. These ointments were stored at room and refrigerator temperatures and assayed from time to time to determine the stability of the penicillin. It was found that the impure sodium penicillin ointment was quite unstable as compared to the calcium salt of similar purity. The crystalline sodium and potassium salts of penicillin were equal to, if not superior to, the calcium salt as far as stability was concerned. The presence of sulfathiazole, sulfadiazine, benzocaine, epinephrine, and phenacaine does not appreciably affect the stability of the sodium, calcium, or potassium penicillin in the ointment bases tried in these experiments. The presence of water, Carbowax, zinc stearate, or dried aluminum hydroxide gel greatly hastens the decomposition of penicillin under the conditions of the experiments.

When crude sodium penicillin first became available in sufficient quantities for various experimental purposes in 1944 it was decided to prepare an ointment of this antibiotic suitable for topical application as well as one for ophthalmic use. It was realized, of course, that the chief problem involved would be one of selecting a base in which the penicillin then available was stable. This was especially important since the resulting product was intended for commercial distribution, which meant that it should lose not more than a relatively small percentage of its potency in a year at refrigerator temperature. Nearly all penicillin ointments and creams mentioned in the literature (1–8) at that time were intended for extemporaneous use because of their relative instability.

Later, when the impure calcium salt of penicillin became available in sufficient quantities for experimental purposes, it was incorporated into bases similar to those used for the impure sodium salt. It was also combined in ointment form with other medicaments such as sulfathiazole, sulfadiazine, phenacaine hydrochloride, benzocaine, and epinephrine.

When the crystalline sodium and potassium salts of penicillin were available they were also prepared in ointment form alone and in combinations with other medicaments.

The ointments prepared for this investigation were made under ordinary laboratory conditions of temperature and humidity. The samples were stored in collapsible tin tubes at room temperature and most of them also at refrigerator temperature. They were assayed by the agar cup-plate method at the time of preparation and at various intervals thereafter. Those in a water-soluble or water-miscible base were first dissolved or shaken with water and an aliquot taken for testing, while those in a grease base were dissolved in ether and the penicillin extracted with a phosphate buffer solution.

EXPERIMENTAL

Stability of Ointments Made with Impure Sodium Penicillin.—Since it is generally accepted that water-soluble constituents in ointment form diffuse more readily from a water-miscible base than from a grease base, it was decided to incorporate crude sodium penicillin into this type of base. Thus one sample was prepared using Carbowax 1500 alone and another using a water-miscible base containing a high percentage of water. Several samples with an anhydrous base containing petrolatum as the chief ingredient were also prepared for comparison in stability studies. The results are shown in Table I.

From the data in Table I it can readily be seen that crude sodium penicillin is much more stable in a nonaqueous base or in one which does not contain Carbowax 1500. However, it is not sufficiently stable in these bases for commercial consideration, but perhaps would be suitable for extemporaneous use.

Stability of Ointments Made with Impure Calcium Penicillin.—When the impure calcium salt of penicillin became available for experimental use, it was tried alone in some of the bases given in Table I, as well as in combinations with other medicaments. The results of these experiments are given in Table II. The oils and petrolatum marked "dried" in this table were heated to about 120° under vacuum for at least two hours and allowed to cool before using to remove any traces of moisture which may have been present.

It is readily seen from the stability data in Table II that calcium penicillin is much more stable in the ointment bases used than the impure sodium salt. Even in the aqueous base or in Carbowax 1500 calcium penicillin is sufficiently stable for prescription use, especially if the ointment is kept at refrigerator temperature. This observation has also been confirmed by others (2–9). It is also specified in the U. S. P. XIII that Penicillin Ointment shall contain only the calcium penicillin.

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TABLE I.—STABILITY OF OINTMENTS CONTAINING IMPURE SODIUM PENICILLIN

Formula		Time	Per Cent of Theoretical Penicillin Potency: Room Temp.	Refrig. Temp.
Penicillin Sodium (400 U./Mg.)	0.1%	0	96	
Carbowax 1500	q. s.	4 days	30	
		8 days	10	
Penicillin Sodium (400 U./Mg.)	0.1%			
Carbowax 1500	25.0%	0	60	
Stenol	25.0%	11 days	15	30
Sodium lauryl sulfate	1.0%	2 mo.		11
Water	q. s.			
Penicillin Sodium (400 U./Mg.)	0.1%	0	75	
Wool fat, anhydrous	5.0%	3 wks.	35	
White Petrolatum	q. s.			
Penicillin Sodium (400 U./Mg.)	0.1%	0	75	
Aquaphor	5.0%	6 wks.	45	
White Petrolatum	q. s.			
Penicillin Sodium (400 U./Mg.)	0.1%	0	87	
Falba Absorption Base	5.0%	2 wks.	42	
White Petrolatum	q. s.			
Penicillin Sodium (400 U./Mg.)	0.1%	0	89	
Peanut Oil	5.0%	2 wks.	36	
White Petrolatum	q. s.			
Penicillin Sodium (400 U./Mg.)	0.1%	0	85	
Corn Oil	5.0%	2 wks.	21	
White Petrolatum	q. s.			
Penicillin Sodium (400 U./Mg.)	0.1%	0	90	
Cod Liver Oil	5.0%	2 wks.	39	
White Petrolatum	q. s.			
Penicillin Sodium (400 U./Mg.)	0.1%	0	100	
Cottonseed Oil	5.0%	2 wks.	46	
White Petrolatum	q. s.			

TABLE II.—STABILITY OF OINTMENTS CONTAINING IMPURE CALCIUM PENICILLIN

Formula		Time	Per Cent of Theoretical Penicillin Potency: Room Temp.	Refrig. Temp.
Penicillin Calcium (650 U./Mg.)	0.1%	0	100	
Carbowax 1500 (dried)	q. s.	2 wks.	7	50
		1 mo.	..	19
Penicillin Calcium (650 U./Mg.)	0.12%	0	93	93
Carbowax 1500	25.0%	11 days	20	71
Stenol	25.0%	18 days	7	54
Sodium Lauryl Sulfate	1.0%	2 mo.	..	19
Water	q. s.			
Penicillin Calcium (500 U./Mg.)	0.1%	0	94	
Cottonseed Oil	5.0%	4 mo.	87	
White Petrolatum	q. s.	6 mo.	75	
		1 yr.	62	
		19 mo.	67[a]	
Penicillin Calcium (500 U./Mg.)	0.1%	0	100	
Mineral Oil	5.0%	1 mo.	100	
White Petrolatum	q. s.	8 mo.	78	
		15 mo.	60	
Penicillin Calcium (650 U./Mg.)	0.1%	0	100	
Sulfathiazole	5.0%	5 mo.	88	90
Cottonseed Oil (dried)	5.0%	9 mo.	88	100[a]
White Petrolatum (dried)	q. s.	15 mo.	60	71
Penicillin Calcium (650 U./Mg.)	0.12%	0	100	
Benzocaine	5.0%	6 mo.	89	95
Cottonseed Oil (dried)	5.0%	1 yr.	84	74
White Petrolatum (dried)	q. s.	15 mo.	72	81[a]
		2 yr.	63	68[a]

(Cont'd on p. 372)

TABLE II.—STABILITY OF OINTMENTS CONTAINING IMPURE CALCIUM PENICILLIN. (*Cont'd from page 371*)

Formula	Time	Per Cent of Theoretical Penicillin Potency: Room Temp.	Refrig. Temp.
Penicillin Calcium (650 U./Mg.).....0.1%	0	100	
Epinephrine.....0.0125%	6 mo.	86	100
Boric Acid.....0.0125%	20 mo.	71	75
Cottonseed Oil (dried).....5.0%	2 yr.	71	75
White Petrolatum (dried)..... *q. s.*			
Penicillin Calcium (650 U./Mg.)..... 0.1%	0	100	
Epinephrine..... 0.0125%	6 mo.	65	96
Boric Acid..... 0.0125%	20 mo.	36	82
Cottonseed Oil (dried)..... ....40.0%	26 mo.	..	65
White Petrolatum (dried)..... *q. s.*			
Penicillin Calcium (650 U./Mg.).....0.12%	0	100	
Phenacaine Hydrochloride.....0.75%	6 mo	61	89
Cottonseed Oil (dried).....5.0%	1 yr.	65	70
White Petrolatum (dried).... ..... *q. s.*	15 mo.	75[a]	89[a]
	2 yr.	47	71
Penicillin Calcium (650 U./Mg.)..... 0.042%	0	100	
Sulfadiazine..... 5.0%	6 mo.	97	100
Cottonseed Oil (dried)..... 40.0%	18 mo.	60	100
White Petrolatum (dried)..... *q. s.*			
Penicillin Calcium (650 U./Mg.)..... 0.1%	0	100	
Peanut Oil (dried)..... 5.0%	6 mo.	79	80
White Petrolatum (dried)..... *q. s.*	16 mo.	61	77
	2 yr.	35	92[a]
Penicillin Calcium (650 U./Mg.)..... 0.05%	0	100	
Peanut Oil (dried).....40 0%	6 mo.	82	85
White Petrolatum (dried)..... *q. s.*	20 mo.	70	94[a]
	2 yr.	35	70

[a] These inconsistencies in assay results may be explained on the basis of uneven distribution of the penicillin in the base or the inherent difficulties in the assay procedure.

The low percentage of oil in the above formulas was included chiefly to aid in the incorporation and uniform distribution of the penicillin as well as other medicaments in the petrolatum. By increasing the percentage of the oil in the base the consistency can be adjusted as desired. Thus the samples containing 40% oil were much thinner than those containing 5% and were intended for ophthalmic purposes.

The inclusion of sulfathiazole, sulfadiazine, epinephrine, phenacaine, and benzocaine did not materially influence the stability of the calcium penicillin in the ointment bases tested.

Temperature of storage, with the exception of the ointment with the aqueous base, did not greatly influence the stability of the calcium penicillin although the assay results of the samples kept in the refrigerator were usually slightly higher than those kept at room temperature.

Stability of Ointments Made with Crystalline Penicillin.—When the crystalline sodium and potassium salts of penicillin became available these were incorporated into some of the ointment bases described above and stored at room and refrigerator temperatures as before for stability studies. The results are summarized in Table III.

From the stability data on the crystalline sodium and potassium salts of penicillin in the ointments listed in Table III it is quite evident that they are equal if not superior to the impure calcium penicillin in the same or similar ointment bases. However, for a fair comparison a calcium salt of similar potency should also be tested. In this connection it is also interesting to observe the great difference in stability of the pure crystalline sodium penicillin as compared to that of the relatively impure sodium salt in ointment form as shown in Table I.

Further Studies on Penicillin Ointments with Water-Miscible Bases.—Since the first experiments with penicillin in bases containing water or Carbowax 1500 showed a very rapid rate of deterioration of the medicament, it was felt that other bases o the water-miscible type should be tried, especiall since this kind of base would be much more desirabl than the grease type.

As most of the solids suitable for such a base ar too firm to be used as such, it was necessary to fin a nonaqueous liquid to give the base an ointment like consistency. Triacetin was found to be suitabl for this purpose. It is somewhat miscible wit water and penicillin calcium was found to be fairl stable when suspended in it, as shown in Table IV Consequently several penicillin ointments were pre pared containing triacetin and other ingredients t give a base of suitable consistency.

The formulas and results of stability tests on thes preparations are given in Table IV. From the re sults of penicillin stability tests in this table it ca be seen that none of these ointments is as stable a those in the petrolatum-type base even when calciu penicillin of fairly high potency or the crystallin sodium or potassium salts are used.

The presence of zinc stearate and dried aluminum hydroxide gel are greatly detrimental to the stability of the penicillin. On the other hand, corn starch and propylene glycol distearate do not hasten the deterioration rate of penicillin. Calcium gluconate is intermediate in this respect.

TABLE III.—STABILITY OF OINTMENTS CONTAINING CRYSTALLINE PENICILLIN

Formula		Time	Per Cent of Theoretical Penicillin Potency: Room Temp.	Refrig. Temp.
Penicillin Sod. Cryst. (1620 U./Mg.)	0.031%	0	99	
Cottonseed Oil (dried)	5.0%	6 mo.	84	83
White Petrolatum (dried)	q. s.	1 yr.	84	84
Penicillin Pot. Cryst. (1583 U./Mg.)	0.032%	0	97	
Cottonseed Oil (dried)	5.0%	6 mo.	73	73
White Petrolatum (dried)	q. s.	1 yr.	70	72
Penicillin Sod. Cryst. (1620 U./Mg.)	0.031%	0	100	
Sulfathiazole	5.0%	6 mo.	98	99
Cottonseed Oil (dried)	5.0%	9 mo.	72	84
White Petrolatum (dried)	q. s.	1 yr.	76	84
		17 mo.	95[a]	100[a]
Penicillin Pot. Cryst. (1583 U./Mg.)	0.032%	0	100	
Sulfathiazole	5.0%	6 mo.	83	98
Cottonseed Oil (dried)	5.0%	9 mo.	76	78
White Petrolatum (dried)	q. s.	1 yr.	76	82
		17 mo.	88[a]	94[a]
Penicillin Sod. Cryst. (1620 U./Mg.)	0.031%	0	100	
Sulfadiazine	5.0%	6 mo.	96	99
Cottonseed Oil (dried)	5.0%	9 mo.	92	98
White Petrolatum (dried)	q. s.	1 yr.	92	87
		17 mo.	94	100[a]
Penicillin Pot. Cryst. (1583 U./Mg.)	0.032%	0	89	
Sulfadiazine	5.0%	9 mo.	84	90
Cottonseed Oil (dried)	5.0%	1 yr.	82	90
White Petrolatum (dried)	q. s.	16 mo.	72	81
Penicillin Sod. Cryst. (1620 U./Mg.)	0.031%	0	95	
Benzocaine	5.0%	6 mo.	94	93
Cottonseed Oil (dried)	5.0%	9 mo.	93	96
White Petrolatum (dried)	q. s.	1 yr.	74	79
Penicillin Pot. Cryst. (1583 U./Mg.)	0.032%	0	100	
Benzocaine	5.0%	6 mo.	97	100
Cottonseed Oil (dried)	5.0%	9 mo.	98	100
White Petrolatum (dried)	q. s.	1 yr.	76	84

[a] These inconsistencies in assay results may be explained on the basis of uneven distribution of the penicillin in the base or the inherent difficulties in the assay procedure.

TABLE IV.—STABILITY OF OINTMENTS OF PENICILLIN IN OTHER WATER-MISCIBLE BASES

Formula		Time	Per Cent of Theoretical Penicillin Potency: Room Temp.	Refrig. Temp.
Penicillin Calcium (845 U./Mg.)	0.1%	0	100	
Triacetin	q. s.	3 mo.	94	
		4 mo.	91	
		7 mo.	37	
Penicillin Calcium (845 U./Mg.)	0.1%	0	98	
Corn Starch (dried)	60%	3 mo.	84	
Triacetin (dried)	q. s.	6 mo.	57	
		12 mo.	24	
Penicillin Calcium (1097 U./Mg.)	0.05%	1 mo.	67	
Corn Starch (dried)	60%	3 mo.	22	54
Carbowax 4000	8%			
Triacetin (dried)	q. s.			
Penicillin Calcium (1097 U./Mg.)	0.05%			
Zinc Stearate	21.0%	0	No activity	
Triacetin (dried)	q. s.			
Penicillin Calcium (1097 U./Mg.)	0.05%			
Dried Aluminum Hydroxide Gel	26.5%	0	No activity	
Triacetin (dried)	q. s.			
Penicillin Calcium (845 U./Mg.)	0.06%	1 mo.	60	89
Lactose (dried)	43.5%	2 mo.	38	82
Triacetin (dried)	q. s.	5 mo.	..	22

(Cont'd on p. 374)

TABLE IV.—STABILITY OF OINTMENTS OF PENICILLIN IN OTHER WATER-MISCIBLE BASES

(*Cont'd from p. 373*)

Formula	Time	Per Cent of Theoretical Penicillin Potency: Room Temp.	Refrig. Temp.
Penicillin Calcium (845 U./Mg.) 0.056%	0	78	
Calcium Gluconate (dried) 46.5%	2 mo.	24	30
Triacetin (dried) ..... *q. s.*	5 mo.	16	21
Penicillin Calcium (845 U./Mg.) 0.06%	0	96	
Corn Starch (dried) 52.0%	2 mo.	56	85
Propylene Glycol Distearate 5.0%	5 mo.	40	84
Triacetin (dried) ..... *q. s.*	8 mo.	8	78
	15 mo.	..	51
Penicillin Sod. Cryst. (1620 U./Mg.) 0.03%	0	78	
Corn Starch (dried) 52.0%	3 mo.	35	77
Propylene Glycol Distearate 5.0%	7 mo.	28	69
Triacetin (dried) ..... *q. s.*			
Penicillin Pot. Cryst. (1583 U./Mg.) 0.03%	0	63	
Corn Starch (dried) 52.0%	3 mo.	59	62
Propylene Glycol Distearate 5.0%	6 mo.	31	62
Triacetin (dried) ..... *q. s.*			

Availability of Penicillin in Various Ointment Bases.—It was desirable to determine the readiness with which penicillin diffused from various ointment bases into the surrounding medium, especially those containing petrolatum in which it was found to be most stable. Consequently bacteriological tests were carried out by placing about 1 Gm. of the ointment being tested on a yeast extract agar plate or cup inoculated with *Staphylococcus aureus*. The zone of inhibition was measured after twenty-four hours at 37°. Table V gives a few examples of the results obtained from these tests.

TABLE V.—AVAILABILITY OF PENICILLIN IN VARIOUS OINTMENT BASES

Ointment	Potency, U./Gm.	Radius of Inhibition Zone on Plate in Mm.	Zone on Cup in Mm.
Calcium Penicillin in Cottonseed Oil (5%) and Petrolatum Base	400	8	8
Calcium Penicillin in Mineral Oil and Petrolatum Base	440	7	8
Calcium Penicillin in Cottonseed Oil (40%) and Petrolatum Base	225	8	10
Calcium Penicillin in Wool Fat, Mineral Oil and Petrolatum Base	150	5	8
Sodium Penicillin in Cottonseed Oil (5%) and Petrolatum Base	825	8	11

It was concluded from the above results that penicillin in a base composed chiefly of petrolatum diffuses from the base into the surrounding aqueous medium. Similar behavior would be expected when the ointment is applied to wounds or to the eye. Other workers (9, 10) have used different means to measure the availability of the penicillin in similar ointment bases and have come to the same conclusions.

CONCLUSIONS

1. Impure sodium penicillin (400 u./mg.) is very unstable in an aqueous or a nonaqueous water-miscible ointment base. It has only limited stability in an anhydrous petrolatum base.

2. Calcium penicillin (500–650 u./mg.) is much more stable than the impure sodium salt in the same or similar bases.

3. The crystalline sodium or potassium salts of penicillin of high potency (1583–1620 u./mg.) are equal, if not superior, to the impure calcium salt for ointment purposes as far as stability is concerned.

4. Penicillin ointments are somewhat more stable at refrigerator temperature than at higher temperatures.

5. The presence of sulfadiazine, sulfathiazole, benzocaine, or epinephrine in a penicillin ointment does not appreciably influence the stability of the penicillin, while water, Carbowax, zinc stearate, and dried aluminum hydroxide gel hasten its decomposition to a marked degree.

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The Effect of Adrenochrome upon Experimental Glycosuria in the Rat*

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Methods for the preparation of adrenochrome and its oxime are described. The effects upon glycosuria of both substances when administered to male rats having pancreatic diabetes and similar rats having alloxan diabetes are reported. The action of adrenochrome under different controlled feeding conditions was determined. This action was demonstrable, but not great. Adrenochrome oxime was without effect under the conditions of the several experiments described.

ADRENOCHROME is an oxidation product of epinephrine. Its preparation was first described by Green and Richter (1). Kisch (2) has reviewed studies of its biological properties. It has been reported that adrenochrome has been used successfully in the treatment of clinical diabetes (3, 4). Snyder, Leva, and Oberst (5) found that adrenochrome and iodoadrenochrome were ineffective in lowering the blood glucose level of normal rabbits, potentiating the effect of insulin, or neutralizing the action of epinephrine.

In the present studies on rats having alloxan diabetes and pancreatic diabetes it was found that large doses of adrenochrome caused a small decrease in the level of glycosuria in force-fed animals and that in alloxan-diabetic rats which ate *ad libitum* there was a more striking decrease in the level of urinary glucose associated with a decrease in voluntary food intake.

METHODS

Adrenochrome.—Adrenochrome was prepared by the method of Buchnea as described by McCarthy (4, 6). In typical preparations 4.58 Gm. (0.025 mole) of epinephrine, was dissolved in a mixture consisting of 125 ml. of absolute methanol and 2.25–2.50 ml. of formic acid, sp. gr. 1.20. To the solution was added 17.33 Gm. (0.075 mole) of silver oxide, and the mixture was stirred mechanically for 50–120 seconds. During this period the reaction mixture was kept at 38–40° by periodic application of an ice bath. At the end of the reaction the silver and silver oxide were removed quickly from the mixture by suction filtration and were washed with 15–25 ml. of fresh absolute methanol. The combined filtrates were cooled in an ice bath for fifteen to thirty minutes. The red crystalline precipitate of adrenochrome was collected by filtration, washed with a little cold absolute methanol and dried on the filter for fifteen minutes under a tube filled with Drierite. The yields obtained in this procedure varied from 0.84 Gm. (18.8%) to 1.28 Gm. (28.6%) depending on the conditions, the highest yield of 28.6% being obtained with 2.5 ml. of formic acid, a reaction time of sixty seconds and a crystallization period of fifteen minutes. Samples of adrenochrome prepared in this manner melted with decomposition, the decomposition point varying with the manner of heating the sample. Decomposition points obtained by dropping small amounts of material on a preheated Fisher-Johns melting-point apparatus were from 132° to 136°. The spectra, in the region 220–600 mμ, of representative samples of adrenochrome in absolute methanol were identical, within limits of experimental error, and, resembling the curve found by Ellis (7) for a cruder product, had maxima at 299 mμ (k = 57) and 479 mμ (k = 25.5). The blood-pressure response in an anesthetized, atropinized dog to an intravenous injection of a typical preparation of adrenochrome was approximately equivalent to only 0.03 μg. of epinephrine per mg. of adrenochrome. A sample for analysis was dried first at reduced pressure over Drierite and finally at 10μ Hg over phosphorus pentoxide at room temperature.

Anal. Calcd. for $C_9H_9O_3N$: C, 60.4; H, 5.06; N, 7.82. Found: C, 59.57, 59.65; H, 4.94, 4.64; N (Kjeldahl), 7.32, 7.37.

Adrenochrome is very soluble in water and methanol and is insoluble in benzene and the less-polar organic solvents. In the presence of moisture it decomposes to a black, water-insoluble, melanin-like substance. In the solid crystalline state some samples were stable for months, as evidenced by the similarity of their spectra and that of freshly prepared adrenochrome, while other samples, presumably somewhat moist, decomposed rapidly.

Adrenochrome Monoxime.—In the preparation of adrenochrome monoxime 1.00 Gm. (5.6 millimole) of adrenochrome was added, with shaking, to a solution of 1.00 Gm. of hydroxylamine hydrochloride and 2.00 Gm. of sodium acetate in 25 ml. of water. The mixture was shaken briefly and allowed to stand for approximately one hour. The bright orange precipitate was collected and twice recrystallized quickly from water, the first time at 0° and the second at room temperature. The final product, dried for thirty minutes on the filter and amounting to 0.40 Gm. (31%), was adrenochrome monoxime dihydrate, m. p. 178–182° dec. after changing from orange to yellow at 97°. Values for the m. p. reported in the literature are: monohydrate, 192° dec. (8); sesquihydrate, 278° (1).

Anal. Calcd. for $C_9H_{10}O_3N_2{:}2H_2O$: C, 46.94; H, 6.13; N, 12.17. Found: C, 46.82, 47.17; H, 5.66, 5.90; N (Dumas), 12.20, 11.94. Elementary analyses and color changes of samples given drying treatment beyond that described above indicated that the anhydrous material was unstable. The

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ultraviolet absorption spectrum of a methanol solution of the dihydrate displayed a fairly sharp maximum at 325 mμ ($k = 65 \pm 7$) and a broad, rather indefinite one at 390–405 mμ ($k = 25$–30). The position of the latter and the exact shape of the spectral curve may depend on the age of the sample.

Feeding Regimen.—Male rats of the Sprague-Dawley strain were maintained on Purina Dog Chow until they reached a weight of 310 Gm. One series of animals was depancreatized by the method of Ingle and Griffith (9). The second series of animals was made diabetic by injecting alloxan intraperitoneally in doses of 25 mg. every other day until glycosuria occurred. In most of the animals 2 doses of alloxan were sufficient to cause some degree of diabetes, but more resistant animals were given as many as 6 injections. After diabetes was established all of the animals were placed in metabolism cages and were fed a medium carbohydrate diet made according to Table I. Some of the animals were force-fed by stomach tube each morning (8:30 to 9:15) and late afternoon (4:15 to 5:00). The techniques and diet were modifications of those described by Reinecke, Ball, and Samuels (10). During the period of adaptation to forced feeding, the amount of diet was gradually increased to prevent the development of "food shock." The animals were brought to a full feeding of 26 cc. (29 Gm.) of diet per rat per day on the fifth day. The food for *ad libitum* eating was placed fresh each morning in clean, glass jars. The amounts of food consumed each day were determined by weighing at the same time each morning. There was no wasting of this fluid diet by the animals.

The animals were kept in an air-conditioned room with temperature at 74 to 78° F. and humidity at 30% to 35% of saturation. Twenty-four-hour samples of urine were collected at the same hour (8:00–8:30 A.M.) and were preserved with thymol. Urine glucose was determined by the method of Benedict (11).

The adrenochrome was made up in water solution and was given by subcutaneous injection either once or twice daily in a volume of 1.0 cc. per injection. The adrenochrome oxime was made up in sesame oil and was given once daily in a volume of 1.0 cc.

EXPERIMENTS AND RESULTS

Experiment 1.—Six rats having alloxan diabetes were treated with 5 mg. of adrenochrome daily for seven days. Five of the 6 rats showed a temporary small decrease in the level of glycosuria, but by the end of seven days the average amount of glucose excreted was as great as during the preinjection period (approximately 4 Gm. per rat per day). These data are not summarized in the charts. During this experiment it was found that adrenochrome is unstable in water solution and that even the powder frequently undergoes some change over a period of several days. It was considered that the failure of the compound to have a more significant and prolonged effect upon the level of urinary glucose may have been due to its instability.

Experiment 2.—Five rats which had alloxan diabetes were treated with 5 mg. of adrenochrome oxime daily for seven days. Five additional rats were treated with sufficient insulin (2 to 12 units daily of crystalline Zinc Insulin, Lilly) to control partially the glycosuria. These animals also received 5 mg. of adrenochrome oxime daily for seven days. In none of these animals was there evidence that adrenochrome oxime modified the level of glycosuria. Larger doses of the compound killed the animals. This compound was relatively stable in powder form. These data are not summarized in the charts.

TABLE I.—MEDIUM CARBOHYDRATE DIET

Constituent	
Cellu flour (Chicago Dietetic Supply)	120 Gm.
Osborne & Mendel salt mixture	40 Gm.
Diet yeast (Pabst)	100 Gm.
Wheat germ oil	10 Gm.
Cod-liver oil	10 Gm.
Vitamin K (2-methyl-1,4-naphthoquinone)	100 mg.
Mazola oil	*200 Gm.*
Casein (Labco)	160 Gm.
Starch	200 Gm.
Dextrin	190 Gm.
Sucrose	200 Gm.
Water to make total of	2000 cc.

Experiment 3.—Adrenochrome was prepared twice each week and the water solution was prepared a few minutes before it was injected into the animal. Six rats having alloxan diabetes were treated with 5 mg. of adrenochrome daily for ten days. All of the rats showed some decrease in glycosuria during the administration of adrenochrome. During the preinjection period the average amount of urinary glucose per rat per day was 2994 mg. It fell to an average of 1770 mg. during the injection period, and increased to an average of 2374 mg. during the postinjection period. These data are not summarized in the charts.

Experiment 4.—Six rats having alloxan diabetes and 6 rats having pancreatic diabetes were treated with 10 mg. of adrenochrome daily (divided doses) for seven days. The adrenochrome was prepared twice each week and was made up in water solution a few minutes before each injection period. All of the rats having alloxan diabetes and 5 of the rats having pancreatic diabetes showed a decrease in glycosuria during the injection period. The average amounts of glucose excreted rose to preinjection levels when the injections of adrenochrome were stopped. These data are summarized in Fig. 1.

Experiment 5.—Twelve rats having alloxan diabetes were studied during *ad libitum* eating. Six rats were treated with 5 mg. of adrenochrome daily for seven days and 6 rats were treated with 10 mg. daily for seven days. The adrenochrome was prepared twice each week and was made up in water solution a few minutes before each injection period. The amounts of food eaten were accurately measured. During the administration of the 5-mg. dose of adrenochrome 4 of the 6 rats showed a significant decrease in food intake and 5 of the animals showed a decrease in glycosuria. During the administration of 10 mg. of adrenochrome daily all of the animals showed a decrease in food intake and a decrease in glycosuria. When the injections were stopped these changes were reversed. These data are summarized in Fig. 2.

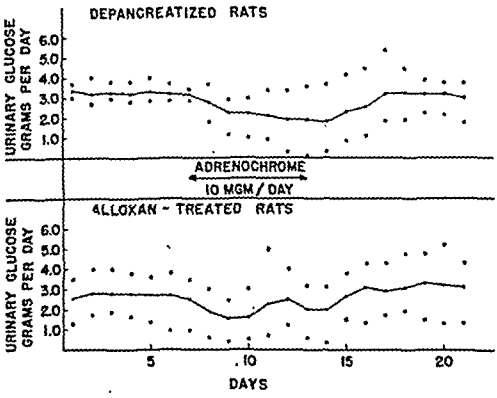

Fig. 1.—The effect of adrenochrome in force-fed, diabetic rats. Averages and range of individual values for glycosuria. Six rats in each of the two groups.

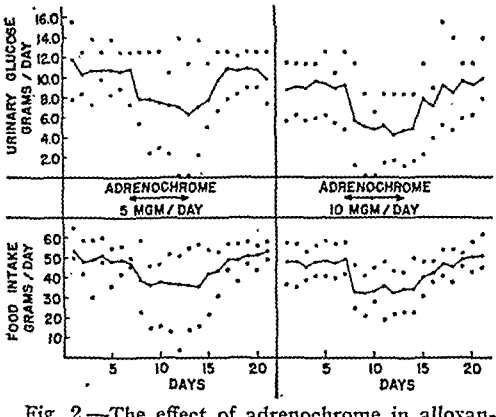

Fig. 2.—The effect of adrenochrome in alloxan-diabetic rats eating *ad libitum*. Averages and range of individual values for glycosuria. Six rats in each of the two groups.

DISCUSSION

It was shown in these experiments that the administration of adrenochrome to diabetic rats can cause a striking decrease in glycosuria, which is secondary to a decrease in voluntary food intake. In addition, there was a small but consistent average decrease in the level of urinary glucose when the intake of food was kept constant by forced feeding. These changes were not striking, but their consistent occurrence under rigid conditions of experiment probably represents a significant response to large doses of adrenochrome.

There have been many reports on compounds and crude natural products having an insulin-like action but in no case has acceptable supporting evidence been offered. It has not been shown that the effect of adrenochrome is related to the action of insulin. Snyder, Leva, and Oberst (5) found adrenochrome to be ineffective in lowering the blood glucose of rabbits. Insulin has a very striking effect upon the glucose load tolerated by the eviscerated rat (9, 12) but we have tested adrenochrome under identical conditions with negative results. Adrenochrome is a toxic compound. It is possible that its small effect upon experimental diabetes is related to its toxicity and is entirely unspecific. Ingle (13) found that injections of 4% formaldehyde caused a decrease in the glycosuria of the force-fed, partially depancreatized rat. The whole problem of the effect of various classes of toxins and of other forms of stress upon diabetes remains to be explored in the force-fed animal.

SUMMARY

Adrenochrome and adrenochrome oxime were tested for an effect upon glycosuria in male rats having pancreatic diabetes and in similar rats having alloxan diabetes. Doses of 5 and 10 mg. of adrenochrome per day caused a small decrease in the level of glycosuria in the force-fed rats. When the glycosuria was partially controlled by insulin the effect of adrenochrome remained slight. The administration of adrenochrome to diabetic rats which ate *ad libitum* caused a more striking decrease in the level of urinary glucose, which was associated with a decrease in food intake. Adrenochrome oxime was without any demonstrable effect in these experiments.

The preparation and some properties of adrenochrome and adrenochrome monoxime are described.

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Microtoxicology. I. Color Reactions of Sympathomimetic Amines*

By THOMAS J. HALEY†

Color reactions by which fifteen sympathomimetic amines may be differentiated and classified are described. These color reactions, in combination with polariscope readings, should enable the toxicologist to identify not only any one of the fifteen sympathomimetic amines but also the isomer present.

THE PREVALENT use of sympathomimetic amines in inhalers, nose drops, tablets, and other forms of medication has led to abuses that are of toxicological importance. Recently there have been two reports dealing with desoxyephedrine toxicity (1, 2). Amphetamine also has caused similar toxic manifestations (3–7).

Means for the identification of the exact amine causing the clinical toxicity have not been adequately developed. It has been shown that the heavy metal alkaloidal precipitants offer a rapid means for differentiating these potent substances (8). Earlier investigators were, in general, interested in the preparation of derivatives of the pure compounds and worked with much larger quantities than are usually available to the toxicologist. In this manner amphetamine has been identified as the benzoyl derivative (9) and *d*-desoxyephedrine as the picrolonate (10).

Inasmuch as the opium and other alkaloids have been identified by their color reactions with various reagents it was decided that a study of the color reactions of fifteen sympathomimetic amines with these reagents should be undertaken.

EXPERIMENTAL

The following alkaloidal reagents were prepared according to the directions in Autenrieth and Bauer (11): Marquis, Frödhes, Rosenthaler-Turk, Mandelin and Meckes. Sanchez reagent (12) was also prepared. To several drops of each reagent on a spot plate or 1–2 cc. of the reagent in a small test tube was added about 1 mg. of the following sympathomimetic amines: *dl*- and *l*-ephedrine; *d*-, *dl*-, and *l*-desoxyephedrine; *d*- and *dl*-amphetamine; Paredrine; Nethamine; Vonedrine; *d*-, *dl*-, and *l*-Neosynephrine; *dl*-Synephrine and Tuamine. The following results were obtained.

Marquis Reagent (Formalin-Sulfuric Acid)

dl-Ephedrine, *l*-Ephedrine — Orange, dark brown, and finally wine colored.

d-, *dl*-, and *l*-Desoxyephedrine, *d*- and *dl*-Amphetamine, Vonedrine — Orange-red, orange-brown, and finally dark brown.

Paredrine—Purple-red and finally dark purple.

Nethamine—Yellow and finally dark brown.

d-, *dl*-, and *l*-Neosynephrine—Rose, red-orange, and finally dark brown.

Synephrine and Tuamine—Colorless.

Frödhes Reagent (Molybdic Acid)

dl-Desoxyephedrine—Weak yellow.

Paredrine—Emerald-blue with a yellow ring.

d-, *dl*-, and *l*-Neosynephrine—Powder blue.

All the other compounds gave colorless solutions.

Mandelin Reagent (Vanadium-SulfuricAcid)

dl- and *l*-Ephedrine—Reddish brown, reddish o stirring.

d-, *dl*-, *l*-Desoxyephedrine—Red, emerald-greei deep green on stirring.

Vonedrine—Yellowish green, emerald-green, dee green on stirring.

d- and *dl*-Amphetamine—Olive-green, greyish gree on stirring.

Paredrine—reddish brown, blackish green on stir ring.

Nethamine—Blood-red, no change on stirring.

d-, *dl*-, and *l*-Neosynephrine, Synephrine — Black, deep green o stirring.

Tuamine—Black, no change on stirring.

Rosenthaler-Turk Reagent (Arsenous Acid)

All the compounds were colorless before heating and after both heating and the addition of concentrated hydrochloric acid a faint yellowish tinge wa noted.

Meckes Reagent (Selenous Acid)

d-, *dl*-, *l*-Desoxyephedrine, Vonedrine, Nethamine — Lemon yellow

Paredrine—Brick red.

d-, *dl*-, and *l*-Neosynephrine—Reddish purple.

Synephrine—Lavender.

Sanchez Reagent (Alcoholic Dimethylaminobenzaldehyde-Sulfuric Acid)

dl- and *l*-Ephedrine, *d*-, *dl*-, and *l*-, Desoxyephedrine, Vonedrine, Tuamine — Emerald-green residue, pale green solution.

d-, *dl*-Amphetamine—Yellowish green residue, yellowish green solution.

Paredrine—Reddish brown residue, yellowish brown solution.

d-, *dl*-, and *l*- Neosynephrine—Orange residue, pale orange solution.

Synephrine—Yellowish brown residue, deep yellow solution.

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DISCUSSION

Marquis reagent enables the investigator to differentiate definitely these amines from other alkaloids because their color reactions are distinctly different (13). However, desoxyephedrine, amphetamine, and Vonedrine react as a group and cannot be distinguished from one another by this test. This is not surprising when one considers their close structural relationship, amphetamine (beta-phenylisopropylamine), desoxyephedrine (beta-phenylisopropylmethylamine), and Vonedrine (beta-phenyl-*n*-propylamine). The compounds most easily distinguished by this test are ephedrine, Nethamine, Paredrine, and the Neosynephrines. All of these give distinctly different color reactions which allow them to be differentiated from one another and the other amines tested. However, this test as well as the other tests employed will not distinguish between the various optical isomers.

Frödhes reagent enables one to identify Paredrine and the Neosynephrines. Both codeine and narcotine give color reactions similar to Paredrine but the presence of the yellow ring with the latter is sufficient to differentiate it from them. The color response of papaverine is similar to that of the Neosynephrines but the former finally becomes yellow whereas the latter retains their blue color.

The nonreactivity of these amines with Rosenthaler-Turk's reagent enables the toxicologist definitely to distinguish them from the opium alkaloids which give definite color reactions with this reagent.

Mandelin's reagent gives distinct color reactions with all of these amines but here again it is impossible to distinguish between optical isomers. This reagent enables the toxicologist to distinguish between such structurally related compounds as amphetamine and desoxyephedrine, which with other reagents give the same color reaction. The colors produced by the amines are sufficiently different from those recorded in the literature (13) to enable them to be distinguished from such alkaloids as papaverine, colchicine, and morphine.

Meckes' reagent gives readily distinguishable color reactions with the Neosynephrines, Paredrine, and Synephrine, which are sufficiently different from those given by other alkaloids to enable them to be identified. The other amines tested gave reactions closely approximating those given by colchicine and veratrine.

Sanchez's reagent is of little value for the differentiation of these amines because of the number of them that give the same color reaction. However, it could be used for the identification of Paredrine, the Neosynephrines, and Synephrine.

When the reactions of the substances tested with these six reagents are considered as a whole, one must conclude that they present a rapid method for classification even though, with the possible exception of Paredrine, individual compounds or their isomers cannot be differentiated. However, by the process of elimination it would be possible to narrow the group down to one or two specific substances. Because these compounds contain an asymmetric carbon atom, the toxicologist could positively identify not only the compound but its exact isomer through its specific rotation.

SUMMARY

A method for the differentiation and classification of fifteen sympathomimetic amines using six common toxicological reagents has been presented. Paredrine and the Neosynephrines give the best color reactions of all the compounds tested. The color reactions reported, in combination with polariscope readings, would enable the toxicologist to identify rapidly not only the toxic agent but also the exact isomer causing the toxic symptoms.

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The Preparation of Tri(Hydroxymethyl)Sulfanilamidomethane*

By GAIL A. WIESE† and JAMES W. JONES‡

Tri (hydroxymethyl) sulfanilamidomethane was prepared by treating tri(hydroxymethyl)-aminomethane with *p*-acetoaminobenzenesulfonyl chloride in an aqueous medium with controlled temperature and subsequent hydrolysis of the acetyl compound. Proof of the presence of three hydroxyl groups in the molecule was accomplished by acetylation and subsequent saponification. The compound was tested *in vitro* against two Gram-negative and two Gram-positive organisms, using sulfathiazole sodium as a standard. In no case was its bacteriostatic action equal to that of sulfathiazole sodium.

THE OBJECT of this investigation was to prepare a "sulfa" drug which would be far more soluble than those now in use without destroying the therapeutic activity inherent to this class of drugs.

In reviewing the literature, it was found a large series of acylamidobenzenesulfonalkanolamides and aminobenzenesulfonalkanolamides were prepared by Adams, Long, and Johanson (1). They established the N^1 alkanol derivatives to be more soluble than their acyl derivatives. Crossley, Northey, and Hultquist (2) reported the preparation of a series of N^1 substituted aliphatic sulfonamides, including several N^1 hydroxyalkyl derivatives. They prepared 2-methyl-2-sulfanilamido-1-propanol and 2-methyl-2-sulfanilamido-1,3-propanol. It was noticed they did not report the preparation of a third compound in this immediate series, namely, tri(hydroxymethyl)sulfanilamidomethane.

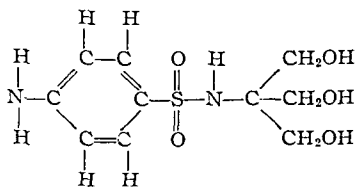
The preparation of tri(hydroxymethyl)sulfanilamidomethane was attempted by three different methods: First, by reacting *p*-acetamidobenzenesulfonyl chloride with tri(hydroxymethyl)aminomethane in an excess of dry pyridine, as described by Goldfarb (3). This reaction progressed smoothly, but difficulty in separating the sulfonamide from the pyridine hydrochloride prohibited the isolation of tri(hydroxymethyl)sulfanilamidomethane. The second attempted synthesis was by the method described by Crossley, Northey, and Hultquist (4), using sodium hydroxide as the condensing agent. As in the previous method, the difficulty of separating the sulfonamide from the reaction mixture prohibited isolation of the compound. The third method, and the one used to obtain the sulfonamide in a pure form, was the method described by Adams, Long, and Johanson (1), involving the use of an additional molecular equivalent of the alkanolamine to act as the condensing agent.

EXPERIMENTAL

Tri(hydroxymethyl)sulfanilamidomethane was prepared by a modified method used by Adams, Long, and Johanson (1). Since the acetyl derivative did not separate from the reaction mixture, it was hydrolyzed within the mixture in the presence of hydrochloric acid. The mixture was then evaporated to dryness under reduced pressure. The mass remaining was broken up with a glass rod, 100 cc. of absolute alcohol added, and the mixture again heated on the water bath until all except the alkanolamine hydrochloride was dissolved. The mixture was cooled, the alkanolamine hydrochloride filtered off and washed with cold absolute alcohol. The filtrate was again cooled and dry ammonia gas bubbled into the alcoholic liquid until a precipitate of ammonium chloride separated which was removed by filtration. The alcoholic filtrate was decolorized with charcoal, then evaporated to about one-fourth its original volume on the water bath. On cooling, the liquid became syrupy in consistency, and on standing in the refrigerator overnight, a precipitate of tri(hydroxymethyl)sulfanilamidomethane formed. The precipitate was collected on the filter and washed with cold 50 % alcohol. The yield of crude product was 60–65% of the calculated. The precipitate was recrystallized once from 50% alcohol and twice from 95% alcohol. On the final crystallization, prisms were obtained which melted at 159 – 161°.

Anal.—Calcd. for $C_{10}H_{16}N_2SO_5$: N, 10.14. Found: N, 10.15.

Tri(hydroxymethyl)sulfanilamidomethane may be represented by the following formula:



One Gm. of tri(hydroxymethyl)sulfanilamidomethane is soluble in 90 cc. of water or 110 cc. of alcohol at room temperature, 25 cc. of hot water and in 20 cc. of hot alcohol. On cooling to room temperature the sulfa derivative did not separate from hot water or hot alcohol. Treatment of tri(hydroxy-

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methyl)sulfanilamidomethane with acetic anhydride and glacial acetic acid gave a penta-acetyl derivative which melted at 193–195°.

Tri(hydroxymethyl)sulfanilamidomethane was tested bacteriostatically, *in vitro*, against four microorganisms, *Proteus vulgaris*, *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus hemolyticus*. A simple qualitative method was used, in which three tubes, each containing 5 cc. of beef infusion, were inoculated with one of the organisms named above. To one of the tubes 25 mg. of tri(hydroxymethyl)-sulfanilamidomethane was added, to another 25 mg. of sulfathiazole sodium, the third tube being used as a control. Tri(hydroxymethyl)sulfanilamidomethane showed inhibition of growth on *Escherichia coli*, *Proteus vulgaris*, and *Streptococcus hemolyticus*, but in no case was the inhibition comparable to that of sulfathiazole sodium. It failed to inhibit the growth of *Staphylococcus aureus*, as did sulfathiazole sodium.

SUMMARY

A new derivative of sulfanilamide was prepared and tested *in vitro* against two Gram-negative and two Gram-positive organisms.

1. Tri(hydroxymethyl)sulfanilamidomethane is more soluble than sulfanilamide, sulfathiazole, and sulfadiazine in either alcohol or water.

2. Tri(hydroxymethyl)sulfanilamidomethane was tested *in vitro* on four microorganisms and found to be less bacteriostatic than sulfathiazole.

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Book Reviews

Vitamins and Hormones, Volume V. Edited by ROBERT S. HARRIS and V. THIMANN. Academic Press, Inc., New York, 1947. xvi + 478 pp. 15 x 23 cm. Price $7.50.

The ever-increasing number of papers on the biological, chemical, and clinical properties of the vitamins and the hormones makes the wisdom of an annual review in these fields apparent.

In Volume V of these reviews, the authors have been carefully selected, as always, to condense and evaluate the most important recent developments.

The recent synthesis of vitamin A makes a review of such synthetic methods advisable. This is ably done by Nicholas A. Milas of Massachusetts Institute of Technology. The question of the physiological availability of the vitamins is reviewed by Doctors Melnick and Oser. These authors also evaluate the limitations of nonbiological methods for determining the vitamins. Other reviews in the vitamin field deal with thiamine and neurophysiology, the effects of pterylglutamates in man, the vitamin requirements of the chick, and the requirements of the mouse.

In the hormone field most of the reviews are of pharmaceutical importance. The reviews consider the properties of the growth and adrenocorticotropic hormones, the effect of exogenous estrogens on the male mammal, the biology of antithyroid agents, the use of androgens in women, and the clinical uses of testosterone in the male.

A valuable feature of the volume is the cumulative index to the first five volumes.

The Aseptic Treatment of Wounds. By CARL W. WALTER, A.B., M.D., assistant professor of surgery at Harvard University, and senior associate in surgery at the Peter Bent Brigham Hospital in Boston. The Macmillan Company, New York. 1947 ix + 372 pp. 21 x 28.5 cm. Price $9.00.

Beginning with a history of the development of the concept of asepsis, and discussing in full detail the various phases of aseptic technique, this book offers a ready reference for surgeons, nurses, pharmacists, and bacteriologists. Other hospital personnel concerned with the necessary space and equipment for carrying out aseptic technique in the hospital will also find this volume valuable. A long list of references and a good index add to its usefulness.

Of particular interest to pharmacists in hospitals is the chapter on the preparation of parenteral fluids in which the author outlines specific procedures for the preparation of solutions. The design of the still, source of water supply, equipment necessary, pyrogen test, cleaning of glassware, and sterilization of solutions are all discussed in detail and diagrammatic illustrations of stills, apparatus, etc., are included. Illustrations of the autoclave along with information on its operation are covered in a separate chapter.

The importance of aseptic technique and how it is accomplished are emphasized throughout this volume, giving special attention to all aspects of the destruction of bacteria in connection with instruments, dressings, and the skin. Routines for carrying out aseptic procedures in the operating room and central supply are outlined in detail along with drawings to illustrate each step.

Air-borne contamination, blood and plasma facilities, control of communicable disease, and maintenance of sterilizing equipment are also covered.

Growth of Plants. BY WILLIAM CROCKER. Reinhold Publishing Corporation, New York, N. Y., 1948. xii + 459 pp. 16 x 23 cm. Price, $10.

The title of this volume does not adequately cover the contribution that the author has made. The book is actually an extensive research review of the many practical problems dealing with the physiology of plants as investigated by the staff of the famous Boyce Thompson Institute for Plant Research. Among more outstanding discussions are those dealing with the physiological effects on plants of many gases, factors affecting dormancy in seeds and buds, a discussion of plant hormones, and the report of the effects on plants of an extensive number of controlled environmental conditions including such factors as artificial light, low temperatures, spectral glass, radiation, and ultraviolet light. Discussions are well illustrated and graphed.

In so far as the book is a critical summary of only those researches carried on at Boyce Thompson Institute it does not cover any one field exhaustively. For example, there is much more general information to be desired than what is covered under research on insecticides, fungicides, plant hormones, and many others. A generous literature citation, however, somewhat makes up for this.

The author stimulates an interest in a variety of plant research problems the results of which are applicable not only to horticulture but also to the public health.—H. W. YOUNGKEN, JR.

Detoxication Mechanisms; The Metabolism of Drugs and Allied Organic Compounds. By R. TECWYN WILLIAMS. John Wiley and Sons, Inc., New York, 1947. viii + 288 pp. 13.5 x 21.5 cm. Price $5.50.

During more recent years pharmacologists have placed greater emphasis on the action of drugs on animals and man than on the action of animal tissues on drugs. Around the turn of the century considerably more research took place which had as its goal knowledge of the metabolism (detoxication) of drugs and organic chemicals in the animal body.

In addition to oxidation and reduction of drugs, animal tissues can bring about certain synthetic and conjugation reactions. The author describes in detail the ability of tissues to form glucuronides, ethereal sulfates, glycine, cysteine, ornithine and glutamine conjugates, and to methylate and acetylate certain substances. Also covered is the ability of animals to convert the highly toxic cyanides into the less toxic thiocyanates.

Compounds covered are the most important aliphatic compounds; aromatic hydrocarbons; phenols; aromatic alcohols, ethers, aldehydes, ketones amides and acids; organic cyanides; aromatic nitro, amino and azo compounds; sulfones, sulfonic acids and sulfonamides; terpenes and camphors; heterocyclic compounds; and organic arsenicals.

This book is a helpful review largely because it shows the great gaps in our knowledge of the metabolism of drugs.

Approaches to Tumor Chemotherapy. Edited by FOREST RAY MOULTON. American Association for the Advancement of Science, Washington, 1947. viii + 442 pp. 18.5 x 26 cm. Price $7.75.

Human cancer is one of the most complex and baffling diseases known to man.

Although many forms of cancer are known to exist they all behave biochemically substantially alike.

The biochemical similarity of cancers coupled with the fact that a few leads have been developed for their chemotherapeutic "cure" offers a new hope for finding a genuine cure or prophylaxis. The papers in *Approaches to Tumor Chemotherapy* were presented at the 1945 Gibson Island Research Conference on Tumor Chemotherapy or were related to the field but presented at the 1946 Conference.

This book is divided into six parts, the first of which deals with a historical introduction. From the five papers comprising Part 2, it is apparent that the field has come a long way in developing a methodology. This is important since any studies become hit-or-miss unless systematic biological methods are developed.

Part 3 deals with the nutritional approach. This approach has proved to be very fruitful in many other fields and it is reasonable to expect that it will be helpful in the tumor field also. Among other subjects considered in this section is the effect of folic acid treatment on various types of tumors.

Part 4 emphasizes the influence of bacterial products on tumors and especially deals with certain polysaccharides which have proved to have the ability to cause the regression of some tumors.

Part 5 consists of seven papers on the nitrogen mustards and Part 6 contains six clinical papers dealing with urethane and oil-soluble organo-metallic compounds, among other things.

The reader will close this book with the realization that tumor chemotherapy poses many more questions than it answers. For the first time, however, there appear points of departure which may lead to spectacular developments in the future.

ERRATUM

In the review of the book entitled *Modern Cosmeticology*, by Ralph G. Harry, which was publ[illegible] the August issue of THIS JOURNAL, page 338, the price was incorrectly stated as $10.00. The corr[illegible] price of this book is $12.00.

Scientific Edition

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The Antihistaminic Drugs*

A Review of the Literature

By THOMAS J. HALEY

THE WIDE interest in the clinical application of drugs which have a blocking action where histamine is concerned has led to many editorials (1–6) and reviews (7–23). These reports have been principally concerned with the older French antihistamine drugs and with two drugs (Benadryl and Pyribenzamine) developed and used in this country. At the present time many other drugs with antihistaminic activity are being synthesized, pharmacologically tested, and therapeutically used in allergic conditions.

Histamine itself has some definite pharmacological effects, some of which are direct and others which are indirect. Table I lists the principal actions of histamine (24).

Dragstedt (25, 26) has pointed out the significance of histamine and/or H-substances liberated in anaphylaxis and a variety of pathological conditions (Futterrehe, allergy, gastroduodenal ulcer). *It is also significant that he pointed out* the antihistaminic action of certain amino acids and suggested that more effective histamine blocking agents could probably be synthesized. Dragstedt compared the physiological effects of histamine with the symptoms observed in asthma, anaphylaxis, and other allergic and pathological conditions and reached the conclusion that although histamine may not always be the main causative agent of these symptoms any prevention of its effects would be of great benefit.

TABLE I.—SUMMARY OF PRINCIPAL ACTIONS OF HISTAMINE

Site of Action	Effect Produced	Some Important Areas Affected
Smooth muscle	Contraction	Bronchiolar, vascular, intestinal, and uterine smooth muscle
Capillaries	Dilatation and increased permeability	Skin and mucous membranes
Glands of external secretion	Secretogogue	Lacrimal, nasal, pulmonary, and digestive glands
Cutaneous endings of pain response	Pain	Skin

This review will attempt to present the available literature of not only those antihistaminic drugs which are in wide use but also those which are just becoming available. A comparison will be made of their chemical structure, pharmacologic action, and clinical usefulness.

* Received March 26, 1948, from the School of Medicine, University of California at Los Angeles, Calif.

CHEMISTRY

Synthesis.—Fourneau and Bovet (27) in 1933 reported the first synthesis of a successful antihistaminic agent, a phenolic ether of the ethanolamine series called 929 F.

Fleming and Rieveschl (33) have reported the synthesis of Benadryl containing radiocarbon (C_{14}). Starting with phenyl magnesium bromide and $C_{14}O_2$ and passing through five intermediates a 70% yield of Benadryl was obtained. This is reduced to a 55% over-all yield when based upon the barium carbonate used to obtain the radiocarbon.

The synthesis of Pyribenzamine, Neoantergan, and a large number of compounds of the ethylenediamine series was reported in 1946 by Huttrer, *et al.* (28). They used three different methods: (*a*) Condensation of the dialkylaminoethyl substituted amino heterocyclic compounds with an alkyl or aralkyl halide (yield 21% to 87%); (*b*) condensation of the halogenated heterocyclic substance with an asymmetrically tri-substituted alkylenediamine (yield 15% to 66%); (*c*) condensation of an alkyl or aralkyl substituted amino heterocyclic derivative with a dialkylaminoethyl halide (yield 78% to 89%). Sodamide or lithium amide was used as the condensing agent.

Clapp, *et al.* (29) have reported on the synthesis of two new antihistamines, N-(2-pyridyl)-N-(5-bromo-2-thenyl)-N-dimethylethylenediamine and its 5-chloro-2-thenyl analog. They condensed 5-bromo-2-thenyl chloride and 5-chloro-2-thenyl chloride with N-(2-pyridyl)-N-,N-dimethylethylenediamine in the presence of either sodium or potassium amide to form the antihistamines. The former was obtained in 54% and the latter in 62% yield. Weston (30) synthesized N-(2-pyridyl)-N-(2-thenyl)-N,N-dimethylethylenediamine by the condensation of N-(2-pyridyl)-N,N-dimethylethylenediamine with 2-thenyl chloride in the presence of sodamide or lithium amide, obtaining a 64% yield.

Burtner and Cusic (31) synthesized diethylaminoethyl-9,10-dihydroanthracene-9-carboxylate and diethylaminoethyl-xanthene carboxylate by interaction of the corresponding acids with diethylaminoethyl chloride in isopropanol solution. Previously they reported the synthesis of diethylaminoethyl fluorene-9-carboxylate (32).

Qualitative Analysis.—Specifications for Pyribenzamine have been reported in New and Nonofficial Remedies (34). This compound gives definite reactions with Reinecke's salt and sulfuric acid. The latter color test enables Pyribenzamine to be differentiated from Benadryl. Identifying tests for Benadryl are also reported in New and Nonofficial Remedies, 1947 (35).

Keenan (36) has shown that both Benadryl and Pyribenzamine may be identified and differentiated by their reaction with chloroplatinic acid. Haley (37) has found that these compounds can be differentiated and identified by their reactions with Mandelin's, Mecke's, Marquis', and Förhde's reagents. Identifying derivatives and the physical constants for several antihistamines are given in Table II.

Quantitative Analysis.—All of the antihistamines which contain nitrogen in their molecules may be analyzed by the Dumas or Kjeldahl methods. Also the bases may be titrated with standard acid and the acid salts with standard alkali but these methods are nonspecific. Pyribenzamine has been analyzed gravimetrically as the dipicrate (34). Benadryl has been analyzed by titration of the free base. Gelvin and McGavack (38) determined Benadryl colorimetrically in blood and spinal fluid using a modification of the Brodie and Undenfriend (39) method for organic bases.

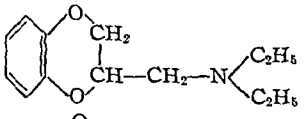
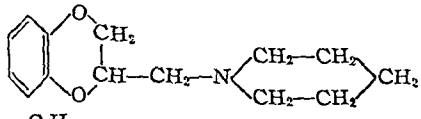
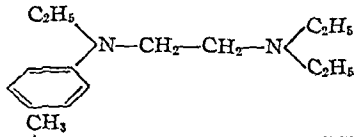
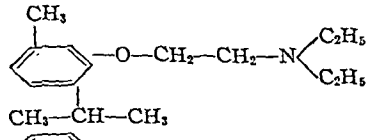
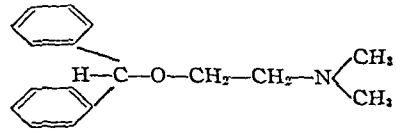
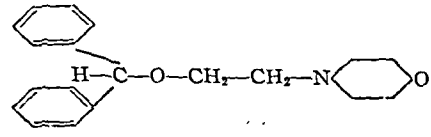
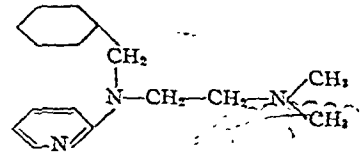
The newer compounds containing sulfur or chlorine may be analyzed after combustion and either of these elements determined by standard methods.

Effect of Chemical Structure on Activity.—When one considers the number of potent antihistamine drugs being used, several facts concerning their structure become evident. The more potent compounds contain as a basic unit either ethanolamine (Benadryl) or ethylenediamine (Pyribenzamine). Further, the terminal N atom should be a tertiary amine, otherwise the potency is of a low order (56). Also it appears that the dimethyl amine derivatives are much less toxic than those containing the diethyl grouping (i.e. Antergan and Benadryl *versus* the Fourneau compounds). However, the terminal N atom may be part of a heterocyclic compound (i.e. Antistine or the morpholine derivative of Benadryl) and have a high antihistaminic value. The chain length between the O and N or the N and N atoms should be not more than 2 C atoms because increased length as well as branching leads to less activity. However, the new phenothiazine compounds do not follow this pattern because one of the most potent compounds, 3277RP, has an isopropyl group connecting the two N atoms, but the cyclic nature of the phenothiazine molecule may be the reason for the activity of 3277RP (47). The change of the aromatic nuclei attached to the alpha N or the ether linkage tends to decrease the activity of the compound (i.e., Benadryl *versus* thymoxyethyldiethylamine or Pyribenzamine *versus* N′-pyridyl-N′-pyridyl-N-dimethylethylenediamine). However, the change from the pyridyl to the pyrimidyl group still leaves the compound with a fair degree of antihistaminic activity. Further, in these same compounds if a para-methoxyl group is added to the benzyl group an increase in antihistaminic activity occurs (Neoantergan or Neoheteramine). Changing the benzyl group to a thenyl or halogenated thenyl group leaves the compound with its original activity (45, 54). Conversion of the compounds related to Benadryl into quaternary ammonium derivatives greatly increases their activity in counteracting acetylcholine but this is to be expected because this structural change makes the compounds more closely resemble choline (52). Similarly the esters studied by Lehmann and Young (44) have a potent acetylcholine blocking action due to their resemblance to the antispasmodic drugs (i.e., Trasentin). Complete evaluation of the structure of the phenothiazine derivatives (47) and of Thephorin (51) in relation to their activity must wait until more information is available regarding structurally related compounds. Another compound, β-pyridylethylmethylamine (PT-9), which has shown great activity in combatting acute histamine cephalalgia may point the way to new compounds which will be specific against this condition as well as migraine (53). A list of some of the more potent antihistaminic drugs is given in Table III.

TABLE II.—DERIVATIVES AND PHYSICAL CONSTANTS OF SEVERAL ANTIHISTAMINES

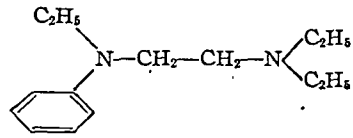
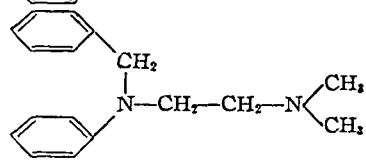
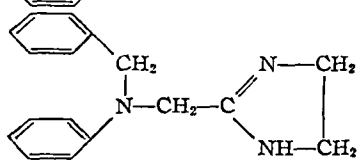
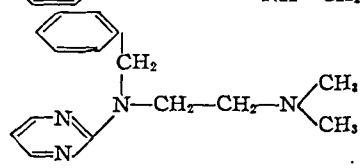
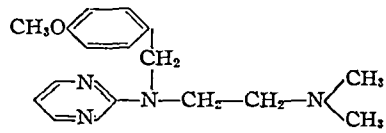
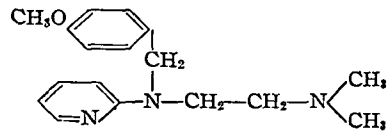
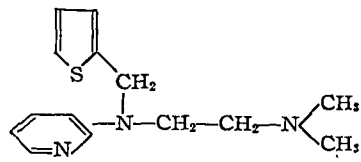
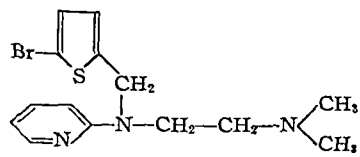
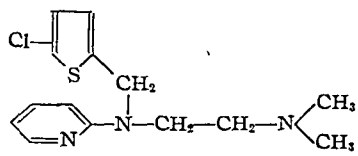
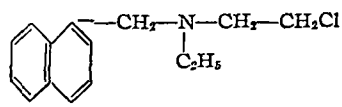
Compound	Derivative	B. P. °C	B. P. Mm.	M. P. °C.	Refractive Index	Reference
Benadryl	HCl	...	...	166–170	...	35
	Di-HCl	...	...	65	...	35
Pyribenzamine	Base	142	0.07	...	1.5759 1.5765	28
	HCl	138	...	193	...	28
	Dipicrate	...	...	185–190	...	34
Histadyl		...	...	161–162	...	..
Thenylene	HCl	...	...	159–161	...	30
Lilly 01013	Base	173– 75	3	...	1.5835	30
	Methiodide	...	...	156–157 d.	...	30
Neoantergan	Base	165–173	0.07	...	1.5750	28
	HCl	...	...	143–143.5	...	28
	Maleate	...	...	100–101	...	98
Diethylaminoethyl-9,10-dihydro-anthracene carboxylate	HCl	...	...	170–171	...	31
Diethylaminoethyl-9, xanthene carboxylate	HCl	...	...	159–160	...	31
Thephorin	Tartrate	...	...	165–167	...	51
Chlorothen	Base	155–175	1	...	...	29
	HCl	...	...	106–108	...	29
Bromothen	Base	173–175	1	...	...	29
	HCl	...	...	124–126	...	29

TABLE III.—CHEMICAL STRUCTURE OF SOME ANTIHISTAMINES

Common Name	Chemical Name	Formula
883 F	Diethylamino-methyl-2-benzodioxane	
933 F	Piperidino-methyl-2-benzodioxane	
1571 F	N′-phenyl-N′-ethyl-N-diethylethylenediamine	
Thymoxyethyldiethylamine Tastromin 929 F	2-Isopropyl-5-methyl phenoxyethyldiethylamine	
Benadryl Diphenhydramin A424 S-51	β-dimethylaminoethyl benzhydryl ether	
Linadryl A 446	β-morpholinoethyl benzhydryl ether	
Pyribenzamine Tripelennamine 63C	N′-pyridyl-N′-benzyl-N-dimethylethylenediamine	

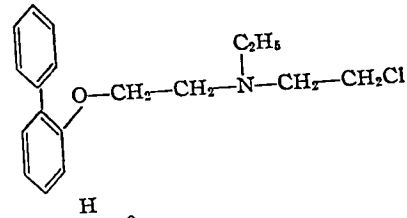
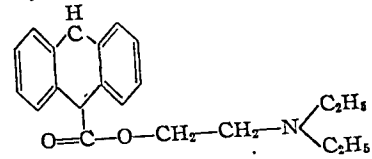
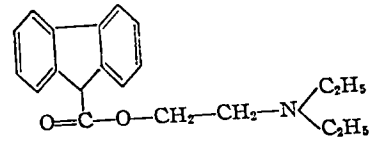
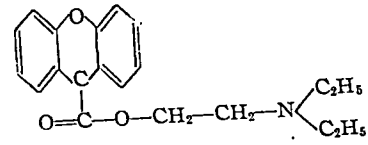
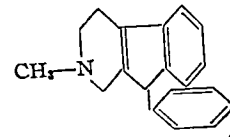
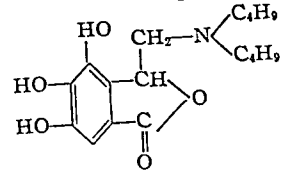
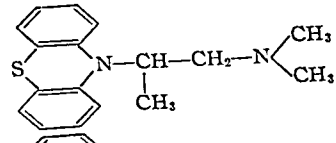
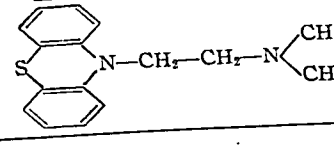
(*Cont'd on p. 386*)

TABLE III.—CHEMICAL STRUCTURE OF SOME ANTIHISTAMINES (*Cont'd from p. 385*)

Common Name	Chemical Name	Formula
2325 RP	N′-phenyl-N′-ethyl-N-dimethylethylenediamine	
Antergan 2339 RP 97 B	N′-phenyl-N′-benzyl-N-dimethylethylenediamine	
Antistine	N′-phenyl-N′-benzyl aminomethylimidazoline	
Heteramine	N′-benzyl-N′-2-pyrimidyl-N-dimethylethylenediamine	
Neoheteramine	N′-*p*-methoxylbenzyl-N′-2-pyrimidyl-N-dimethylethylenediamine	
Neoantergan Pyranisamine Anthisan 2786 RP	N′-*p*-methoxybenzyl-N′-pyridyl-N-dimethylethylenediamine	
Thenylene Histadyl Methapyrilene AH-42 Lilly-01013	N′-pyridyl-N′-thenyl-N-dimethylethylenediamine	
Bromothen	N′-pyridyl-N′-5-bromothenyl-N-dimethylethylenediamine	
Chlorothen	N′-pyridyl-N′-5-chlorothenyl-N-dimethylethylenediamine	
	α-Naphthylmethylethyl-β-chloroethylamine	

(*Cont'd on p. 387*)

TABLE III.—CHEMICAL STRUCTURE OF SOME ANTIHISTAMINES (*Cont'd from p. 386*)

Common Name	Chemical Name	Formula
	β-2-biphenoxyethyl-β-chloroethylethylamine	
D	Diethylaminoethyl-9,10-dihydroanthracene carboxylate	
Pavatrine F	Diethylaminoethyl-7-fluorene carboxylate	
X	Diethylaminoethyl-9-xanthene carboxylate	
Thephorin Nu 1504	2-Methyl-9-phenyl-2,3,-4,9-tetrahydro-1-pyridindene	
Anthallan	3′-Di(*n*-butyl)aminomethyl-4,5,6-trihydroxybenzo-(1,2c)furan-1′-(3′)-one	
3277RP	N-dimethylamino-isopropylthiodiphenylamine	
3015 RP	N-dimethylaminoethylthiodiphenylamine	

GENERAL PHARMACOLOGY

Effect on Blood Pressure and Respiration.—Winder and Thomas (57) have shown that Benadryl intravenously given in phenobarbitalized dogs at a rate of 1–43 mg./Kg. per minute (1–16 mg./Kg.) produced a primary vascular depression of about two minutes' duration. This blood pressure fall varies from 0.05% to 19% of the previous level. A secondary rise in blood pressure of about forty-six minutes' duration occurred after the primary fall but was often masked by the primary depression. Both phases were found to be independent of vagal cardiac innervation, important arterial reflex zones, heart rate changes, peripheral cholinergic mechanisms, and nervous or mechanical respiratory changes. Variable slight cardiac acceleration, independent of the blood pressure changes, was observed with the low doses, while larger doses showed a primary deceleration. Electrocardiographic records showed no gross disturbances in cardiac rhythm. In doses of 50 μg., Benadryl caused a slight to moderate decrease in the

amplitude of systolic contractions of the perfused rabbit heart; at the same time a transient increase followed by a persistent mild decrease was observed in the coronary flow. It was shown that the rate of injection determined the type of respiratory response seen. At the rates of 1.2 to 43 mg./Kg. per minute a slight to moderate, transient stimulation of respiration occurred, while at rates of 19 or more mg./Kg. per minute an early restriction in depth of respiration occurred. Larger doses caused an acceleration or intense polypnea lasting over an hour. The restriction in depth and most of the acceleration in respiration were eliminated by vagotomy and when the carotid innervation was also removed, respiration was not disturbed by large doses. Previously Page and Green (58) found that dogs under pentobarbital anesthesia responded to intravenous Benadryl with a sharp fall in blood pressure and a cessation of respiration. However, pulse pressure remained full despite the low blood pressure. They also observed that there was a reduction in the pressor responses of tyramine, epinephrine, barium chloride, angiotonin, and histamine after Benadryl. In the perfused isolated rabbit ear a greatly diminished vasoconstrictor response was seen when these drugs were injected with Benadryl. In both of these experiments epinephrine was difficult to block. Loew, MacMillan, and Kaiser (59) have shown that epinephrine is potentiated by Benadryl. After intravenous doses of Benadryl (3 mg./Kg.), 10 μg. of epinephrine gave an average response of 64 mm. Hg, whereas the same dose without premedication gave an average response of 45 mm. Hg. However, Benadryl had a depressor action in larger doses, and doses of 1 mg./Kg. gave decreases of 5–10 mm. Hg and doses of 3 mg./Kg. gave decreases of 10–30 mm. Hg. Dose of 1 mg./Kg. had no effect on respiration and doses of 3 mg./Kg. increased the rate 20–40% usually for a period of one to three minutes.

Yonkman, *et al.* (60) have shown that Pyribenzamine intravenously in doses of 10–200 μg./Kg. caused minor fluctuations in cats' blood pressure after the small doses and a sustained rise after the larger doses. They also found that Pyribenzamine potentiated epinephrine. Previously Yonkman, *et al.* (61), found that intravenous Pyribenzamine produced variable degrees of hypotension in normal dogs.

Lee, Dinwiddie, and Chen (45) observed that Thenylene administered intravenously in cats at doses of 1 mg./Kg. caused a very slight hypotension followed by an elevation in the blood pressure. The drug also potentiated the pressor action of epinephrine. A slight decrease in the depth of respiration was also seen. This blood pressure effect has been confirmed (62).

Loew, Micetich, and Achenbach (63) showed that benzhydryl-ethyl-β-chloro-ethylamine intravenously in cats and dogs in doses of 10 mg./Kg. reversed the pressor response of epinephrine. Further, a 10 mg./Kg. dose of this drug to unanesthetized dogs prevented the hypotension and/or the pressor response to epinephrine for seventy-two hours. Similar results were reported for another drug of this series, alpha-naphthylmethylethyl beta-chloroethylamine (64). These epinephrine pressor reversal and blocking effects are similar to those originally reported for the first antihistamine compounds.

Halpern (42) reported that 2325 RP in doses of 10 mg./Kg. caused a rise in blood pressure of 20–60 mm. with a duration of five to twenty minutes. Antergan in a dose of 5 mg./Kg. under the same conditions had no effect. In chloralose dogs, both compounds produced apnea, then respiratory acceleration. Acceleration and a more profound respiration was observed in unanesthetized dogs. Neither compound modified the effect of epinephrine on cardiac tissue. Parrot (65, 66) has confirmed the hypertensive action of Antergan in both dogs and cats.

Bovet, Horclois, and Fournel (67) found that intravenous doses of Neoantergan 1–5 mg./Kg. produced a slight rise in the dogs' blood pressure, larger doses caused hypotension and doses of 50 mg./Kg. death by cardiovascular collapse.

Lehmann and Knoefel (68) have investigated diethylaminoethyl-fluorene-9-carboxylate and found that doses of 0.05 mg./Kg. intravenously in dogs had no effect upon either blood pressure or respiration. In the spinal dog doses of 2 mg./Kg. gave the same results. It was 130 times less effective than atropine in counteracting the depressant effect of acetylcholine in the dog. In further work with this compound and with diethylaminoethyl-xanthene carboxylate and diethylaminoethyl-9,10-dihydroanthracene-9-carboxylate (69) they confirmed their previous work on the first compound, and showed that the dihydroanthracene compound had a hypotensive effect at a dose of 0.5 mg./Kg. This compound also reduced and at times reversed the pressor effect of epinephrine in etherized cats.

Minard and Rosenthal (80) reported that subcutaneous injection of 40 mg./Kg. of thymoxyethyldiethylamine caused an increase in pulse and blood pressure as well as a transitory rise in hemoglobin level. The peak occurred about thirty to forty-five minutes after injection and there was a gradual return to normal.

Anthallan has only a slight vaso-pressor effect in animals in small doses but doses approaching the LD_{50} cause a profound blood pressure fall. Respiration is stimulated only with large doses (81).

Dreyer and Harwood (82) reported that Neoheteramine (1–5 mg./Kg.) caused an immediate fall in blood pressure without a change in heart rate in atropinized cats and dogs under urethane or urethane and chloralose anesthesia. In the Straub heart Neoheteramine caused diminished systolic contractions. Cardiometric measurements in the dogs confirmed this action of the drug. Slight transient dilatation of the perfused rabbit's ear was also seen after Neoheteramine. No potentiation of epinephrine response could be demonstrated on either the blood pressure of the cat or on the isolated uterus of the rabbit.

Reuse (130) observed an increased coronary flow and a depression of the force and rate of beat in the perfused cat heart due to Antergan, Neoantergan, 3277 RP, and Benadryl. Similar results were obtained with the isolated rabbit heart.

Autonomic Effects.—Dreyer (83) reported that, in cats, 1.5 mg./Kg. of Benadryl intravenously caused a diminution of salivary flow on chorda tympani stimulation but not on cervical sympathetic ganglion stimulation. The former was completely blocked with a dose of 5 mg./Kg. but the latter only partially blocked. Motor response to vagal stimulation also was partially blocked.

Yonkman, *et al.* (60, 61), found that Pyribenzamine blocked the salivary secretogogue effect of histamine but had no effect on salivation produced by pilocarpine, epinephrine, or stimulation of the chorda tympani or the cervical sympathetic ganglion. Further, Pyribenzamine potentiated the effect of epinephrine on the nictitating membrane of the cat.

Halpern (42) reported that 2325 RP and Antergan both increased salivary secretion and decreased urinary secretion in dogs. Also neither compound modified the effect of epinephrine or acetylcholine on cardiac tissue or on the autonomic nervous system.

Chauchard and Chauchard (86) showed that Antergan, 2.5% applied locally, decreased the chronaxia of the cervical sympathetic system and at times blocked conduction. Upon the post-ganglionic fibers of that system the reverse was true.

Neoheteramine has no effect on the blood pressure response elicited by sympathetic stimulation in the cat. Some blocking of the chorda tympani response in cats was seen but with larger doses (8 mg./Kg.) did not abolish the secretion. Neoheteramine partially prevented electrically induced vagal stimulation in the cat but had no effect upon the vagal effects on the intestine. At times intestinal vagal contractions were potentiated by the drug (82).

Effect on the Intestinal and Genitourinary Systems.—Loew, *et al.* (59), reported that while Benadryl and the quaternary ammonium derivative of Benadryl were effective in antagonizing the spasmogenic action of acetylcholine on the isolated guinea-pig ileum they were far less effective than atropine in this action. Against barium-induced spasm both compounds were less effective than Pavatrine or Trasentin. 1571 F under the same conditions was comparatively impotent. Sherrod, Schloemer, and Loew (70, 71) found that when equal doses (3 mg./Kg.) of Benadryl, Pyribenzamine, or Neoantergan were administered intravenously to dogs there was a reduction in tone and an inhibition of the spontaneous activity of the duodenum with the former and an increase in duodenal tone with the latter two compounds. The duration of action was about thirty minutes regardless of the drug given. As acetylcholine had little or no effect on the duodenum *in situ* there was no possibility of demonstration of an *in vivo* anticholinergic effect by the three drugs. None of the drugs had any effect on the uterus *in vivo*. Schild (136) found Benadryl more potent than Neoantergan in counteracting acetylcholine spasm of the isolated guinea pig ileum. However, if the drugs were left in contact with the tissue the latter compound showed a greater potency.

Hoekstra and Steggerda (72), using thorium dioxide as an opaque medium and a tambour system, found that intracardial Pyribenzamine (0.1–2.9 mg./Kg.) increased the amplitude and frequency of colonic activity in proportion to the amount administered. In these experiments with six dogs an increase in intestinal muscular tone was also observed. Kaiser and Reynolds (73) reported that there was an increase in the frequency and duration of the contractions of the endometrial blood vessels of intraocular transplants in the anterior chamber of the rabbit after Pyribenzamine. The drug was administered by instillation into the conjunctival sac. The effect observed could have been due to a milking action rather than to any estrogenic effect of Pyribenzamine.

Thenylene has a very weak antispasmodic action on the rabbit intestine when compared with papaverine against barium chloride and atropine against acetylcholine (62). Lee, *et al.* (45), observed that 1:50,000 to 1:20,000 concentrations of Thenylene caused contraction of the virgin guinea-pig uterus. Further, they found that on the guinea-pig intestine the drug antagonized Mecholyl but had only about $^1/_{200}$ the effect of atropine.

Halpern (42) reported that 100 gamma of 2325 RP slightly relaxed the isolated rabbit intestine without affecting its rate of contraction while the same dose of Antergan was similar to epinephrine in its effect. Both compounds caused contraction of the isolated guinea-pig uterus. Mayer (79) found Antergan and Pyribenzamine about equal in counteracting the acetylcholine induced spasm of the guinea-pig ileum *in vitro*.

Lehmann and Knoefel (68) found that diethylaminoethyl-fluorene-9-carboxylate in doses of 1.12×10^{-7} to 8×10^{-8} counteracted acetylcholine 10^{-6} when tested on the isolated ileum. The drug also counteracted barium 10^{-4} in doses of 8×10^{-6} to 4×10^{-6}, and had a relaxant effect upon the dog and rabbit intestine *in vivo* at doses of 0.1 mg./Kg. and 0.05 mg./Kg. The normal dog uterus and the detrusor of the urinary bladder were relaxed *in situ* with doses of 2 mg./Kg. The guinea-pig ileum *in situ* was also relaxed. Further work on this drug and two others, diethylamino-xanthene carboxylate and diethylaminoethyl - 9,10 - dihydroanthracene - 9 - carboxylate, showed that the anthracene compound was more effective than either of the others against acetylcholine spasm. The xanthene derivative was less effective than the others against barium spasm of the rabbit ileum. The anthracene compound alters the effect of epinephrine on the isolated rabbit and human uterus. Both the xanthene and the anthracene compounds relaxed the rabbit ileum *in situ* (69). Lehmann (74) studied the anthracene and fluorene compounds further and found that the rabbit uterus *in situ* and *in vitro* was relaxed by doses of 3–5 mg./Kg. of the former while the latter caused contraction. Upon the isolated human uterus both compounds caused relaxation of both the circular and longitudinal muscles.

Rosenthal and Brown (78) found that thymoxyethyldiethylamine 2 mg. reduced but did not abolish the intestinal spasm of guinea-pig ileum caused by acetylcholine 0.01 mg.

Anthallan stimulates the isolated intestine and uterus in high doses. No gastrointestinal symptoms (nausea, vomiting, diarrhea, stool consistency or frequency) were observed even in lethal doses (81).

Reuse (130) showed that Neoantergan and Antistine were about equal in counteracting acetylcholine spasm of the guinea-pig ileum. Further, both drugs were more potent than 3277 RP. Neoantergan and 3277 RP had no effect on the isolated nonpregnant cat uterus and both drugs counteracted acetylcholine but at higher doses than were required to counteract histamine. Similar results were obtained on the pregnant and nonpregnant rabbit uterus and virgin guinea-pig uterus with Neoantergan, 3277 RP, Antistine, and Benadryl.

Lehmann, *et al.* (75), reported that Thephorin counteracted the spasmogenic effect of acetylcholine and barium on the isolated guinea-pig intestine.

Shock.—Ingraham and Wiggers (85) showed that Benadryl 15 mg./Kg. subcutaneously thirty minutes before hemorrhage and 5 mg./Kg. as a second dose one hour later had no effect on hemorrhagic shock in dogs. All the criteria of irreversible shock were seen and the authors concluded that histamine plays no part in the irreversible tissue damage of hemorrhagic shock.

Jourdan and Chatonnet (84) found that 10 mg./Kg. of Antergan had no effect on the development of experimental traumatic shock and sometimes made it worse.

Anesthetic and Analgetic Effects.—Reuse (130) has shown that 3277 RP, Benadryl, Neoantergan, and Antistine produced local anesthesia in the frog lumbar plexus preparation and that the first two compounds were the most potent.

Halpern (42) found that 2325 RP had a local anesthetic action in animals.

Dews and Graham (97) reported that Neoantergan had a slight analgetic effect in rats in doses of 12 mg./Kg. and that when the dose was increased to 100 mg./Kg. a greater analgesia as well as a narcotic effect was seen. Graham (94) showed by the guinea-pig wheal test that Neoantergan was 3.3 times as strong, Benadryl 2.5 times as strong, and Antistine 1.5 times as strong as procaine.

Lehmann and Knoefel (68) found that diethylaminoethyl-9-fluorene carboxylate and diethylaminoethyl - 9,10 - dihydroanthracene-carboxylate were equal in anesthetic value when tested on the rabbit cornea. Diethylaminoethyl-xanthene-9-carboxylate also has an equivalent local anesthetic effect (69).

Burchell and Varco (127) reported that large subcutaneous doses of thymoxyethyldiethylamine lowered sensitivity to pain but did not produce complete analgesia in dogs.

Effect on the Excised Trachea.—Castillo and de Beer (76, 77) have shown that Benadryl, $1:15 \times 10^6$, counteracted acetylcholine induced contraction of the normal excised guinea-pig tracheal chain. However, Benadryl had no effect on the normal tracheal chain nor did it have any effect upon barium-induced contraction.

Acute Toxicity.—In general, all of the antihistamines present the same train of symptoms when administered in toxic doses to various animals. Benadryl and its β-morpholinoethyl derivative produced violent excitement, convulsive attacks, prostration, and death by respiratory and cardiac failure (50, 88). In dogs an intravenous dose of 5 mg./Kg. caused a slight spasticity, apprehension, and occasionally a clonic convulsion of short duration. The morpholine derivative responded similarly at a 15 mg./Kg. dose but not at lower doses. Violent excitement, prostration, and convulsions were caused by both compounds at a dose of 20 mg./Kg. Other symptoms seen were dilated pupils, exaggerated somatic reflexes, increased sensitivity to sound, and painful sensations of the skin and feet. The reactions of all animals were the same regardless of the route of administration but the onset varied in rapidity. However, in dogs Benadryl had a longer duration of action than the morpholine compound. Although tolerance did not develop in dogs with daily doses of 10 mg./Kg. of Benadryl for twelve days the duration of the symptoms decreased. Sedation with barbiturates thirty to ninety minutes before intravenous injection of either compound prevented excitement and convulsions but did not lessen respiratory-cardiac depression or decrease the mortality (87).

Pyribenzamine presents the same preliminary symptoms in most animal species but at times a secondary depression intervened between excitement and death. In dogs surviving intravenous doses of 3 mg./Kg. the marked excitement and salivation gradually decreased until recovery. Local necrosis of the skin was seen in the rats surviving subcutaneous doses and fibroblasts replaced the muscle cells of the gastroenemii of rats injected intramuscularly (90). The castration of female rats brought their LD_{50} values up to those of the males. Estrogen therapy did not appear to cause a return to greater vulnerability (90, 91).

Neter (93) showed that Benadryl, Pyribenzamine, and Heteramine were toxic to embryonic chick hearts. Benadryl and Pyribenzamine in amounts of 2.5 mg. caused immediate bradycardia and stoppage within two minutes. Hemorrhage was sometimes seen at this concentration but when the concentration was decreased (0.1 to 0.0001 mg.) there was no effect. Heteramine had a similar action depending upon the concentration used.

Halpern (42) reported that toxic doses of both 2325 RP and Antergan caused psychomotor excitement, tetanic convulsions, polypnea, locomotor ataxia, opisthotonis, and death by asphyxia. In dogs the psychomotor effects last from 0.5 to 2 hours.

Graham (94) compared the toxicity of three antihistamines and found that Neoantergan acted at once to cause generalized tremor, increased activity, incoordination of movement, Straub tail phenomenon, squeaking, restlessness, clonic convulsions, and death by asphyxia. Benadryl was similar but less violent; no tail phenomenon was observed but incoordination and excitement were of longer duration and death occurred from general exhaustion. Antistine caused slight motor incoordination, weakness, and marked depression which usually ended with asphyxial convulsions due to depression of vital centers and respiratory paralysis.

Litchfield, *et al.* (54), compared the acute toxicity of Chlorothen, Bromothen, Thenylene, and Pyribenzamine and reported the following symptoms: excitement, tremors, convulsions, tonic and clonic muscle spasms, and respiratory irregularities. At a dose of 2 mg./Kg. intravenously every twenty minutes, a dog showed some of the above toxic symptoms and died after receiving a total of 18 mg./Kg. of Pyribenzamine. Bromothen at twice the dose produced a similar effect but the death did not occur even after a total dose of 36 mg./Kg. See Table IV for the LD_{50} of the various compounds.

Chronic Toxicity.—Gruzhit and Fishen (50) studied the chronic toxicity of Benadryl and its morpholine derivative. They found that mice gained weight at the same rate as the controls when they were fed the morpholine compound as 0.1% of their diet, whereas the Benadryl-fed mice did not. Greater percentages of both compounds caused weight losses in all the mice. Rats gained weight when Benadryl up to 0.25% of the diet was fed but above that level there was a weight loss. Dogs given both compounds in single daily doses of 10 mg./Kg. showed a slight fluctuation in body weight, but there was no disturbance in their hematology or total blood NPN; also the urine was free of sugar

TABLE IV.—LETHAL DOSES OF VARIOUS ANTIHISTAMINIC DRUGS

Drug	Animal	Route Given	LD_{50} ± S. E., Mg./Kg.	Reference
Benadryl	Mouse	Oral	164	50
			270	51
			167	89
	Mouse	Subcutaneous	127	50
			130	89
	Mouse	IP	98	50
			82	89
			86	51
			140 ± 13	160
	Mouse	IV	27.5	51
			18.26 ± 0.6	62
	Rat	Oral	500	50
			545	89
	Rat	Subcutaneous	474	50
	Rat	IP	61 ± 9.0	88
	Rat	IV	42	50
			45.7	89
	Rabbit	IV	10	50
			10.5	89
			17	89
	Guinea Pig	IP	125	51
	Dog	IV	24	50
			30	50
β-morpholinoethyl benzhydryl ether A446	Mouse	Oral	327	50
		Subcutaneous	440	50
		IP	185	50
	Rat	Oral	916	50
		IV	35	50
	Rabbit	IV	21	50
	Dog	IV	70	50
Pyribenzamine	Mouse	Oral	210	94, 51
			200 ± 14.0	141
	Mouse	Subcutaneous	75	92
	Mouse	IV	12	92
			9	51
			29.7 ± 1.67	62
	Mouse	IP	82	51
			62 ± 7.0	141
			68 ± 7.0	160
	Rat M	Oral	570	92
	F	...	515	92
	Rat M	Subcutaneous	340, 350	92
	F	...	225, 250	93
	Rat M	IV	12	92
	F	...	12	92
	Rabbit	Subcutaneous	33	92
		IV	27 ± 3.0	141
		IV	9	92
	Dog	IV	3–4	92
		Oral	20–30	141
Thenylene	Mouse	Oral	182.2 ± 12.8	62
			190	63
	Mouse	Subcutaneous	60	63
	Mouse	IP	90	63
			79 ± 12	141
	Mouse	IV	13	63
			19.85 ± 0.69	62
	Rat	Oral	450	63
		IP	70	63
	Guinea Pig	Oral	250	63
		Subcutaneous	25	63
			70	63
	Cat	Oral	150	63
		IP	55	63
	Dog	Subcutaneous	25	63
		IV	10	63
2325 RP	Rat	Oral	115	66
		IV	50	66
	Dog	IM	20–25	66
		IV	10	66

(Cont'd on p. 392)

TABLE IV.—LETHAL DOSES OF VARIOUS ANTIHISTAMINIC DRUGS (*Cont'd from p. 391*)

Drug	Animal	Route Given	LD_{50} ± S. E., Mg./Kg.	Reference
Antergan	Rat	Oral	30	66
		IV	17.5	66
	Dog	IM	10	66
		IV	5	66
Neoantergan	Mouse	IV	30	94
		IP	102 ± 11	160
Diethylaminoethyl-9,10-dihydroanthracene carboxylate	Mouse	IP	150	70, 71
Diethylaminoethyl-9, xanthene carboxylate	Mouse	IP	250	71
Diethylaminoethyl-9, fluorene carboxylate	Mouse	IP	320	70
	Rat	IP	211 ± 19	88
Anthallan	Mouse	Oral	3200–2700	82
		Subcutaneous	1300	82
		Rectal	650	82
		IP	285	82
		IV	77	82
	Guinea Pig	Oral	2700–2750	82
		Subcutaneous	600	82
	Rabbit	Oral	2000	82
	Cat	Oral	2000	82
		IV	50	82
	Dog	Oral	2325	82
		IV	83	82
	Monkey	Oral	100	82
Heteramine	Mouse	Oral	300–400	91
		Subcutaneous	62.5–75	91
		IP	76 ± 7	160
Thephorin	Mouse	Oral	280	141
		Subcutaneous	300	141
				141
		IP	88	141
		IV	225	141
	Rat	Oral	280	51
	Guinea Pig	IP	125	51
	Rabbit	Oral	500	51
		IV	17	51
	Dog	...	33	51
Antistine	Mouse	Oral	150	51
Chlorothen	Mouse	Oral	390 ± 27	141
		IP	112 ± 10	141
	Rabbit	IV	77 ± 14	141
Bromothen	Mouse	Oral	430 ± 25	141
		IP	130 ± 6	141
	Rabbit	IV	79 ± 7	141
	Dog	Oral	90–120	141
3015 RP	Mouse	IP	140 ± 13	160
3277 RP	Mouse	IP	190 ± 21	160

and albumin. Some acute toxic phenomena (emesis, irritability, etc.) were seen with doses of 25 and 40 mg./Kg. of Benadryl while the morpholine compound caused a slight lowering in the red cell count and hemoglobin values at 40 mg./Kg. A dose of 60 mg./Kg. of the latter gave results similar to the 40 mg./Kg. dose of the former. Weight loss, persistent anorexia, decreased red cell counts and hemoglobin values, and mild albuminuria were seen when 80 mg./Kg. of the morpholine compound was given. However, total blood NPN remained normal. Daily subcutaneous injections for twenty-one days of 2.5, 5.0, and 7.5 mg./Kg. of Benadryl caused a weight loss in guinea pigs. Both compounds upon prolonged administration to small animals produced cachexia, gastrointestinal atony, congestive stasis of visceral organs, and superimposed inflammatory foci in the lungs. The thyroid glands showed mild depletion of colloid substance and a mild hypertrophy of the acinal cells. Large animals showed no parenchymatous tissue changes. Chen and Abreu (89) found that CCl_4 poisoned rats detoxified Benadryl (141 mg./Kg.) in the same manner as the normals.

Rieveschl and Gruhzit (88) reported that Benadryl administered orally in doses of 10, 25, 40, and 60 mg./Kg. per day to mice caused no changes in blood counts, hemoglobin values, NPN values, no change in eating habits or any significant weight change. Doses of 10–25 mg./Kg. in dogs produced no acute or chronic degenerative changes in the liver, spleen, kidney, pancreas, adrenals, heart, brain, gall bladder, urinary bladder, or gastrointestinal mucosa.

Further, there was no congestion, edema, petechial hemorrhages, or necrotic cells in the central nervous system tissues.

Koepf, Arbesman, and Munafo (95) reported that Pyribenzamine administered orally to dogs in doses of 50 to 100 mg. daily for a period of one year had no effect upon body weight, kidney function, liver function, blood urea nitrogen, blood creatinine values, hematocrit, hemoglobin values, red cell, white cell, and polymorphonuclear leucocytes. Mayer, *et al.* (90), working with rats of both sexes reported the same results after the oral administration of 5 mg./Kg. per day for five months. Further, they found no changes in appetite, reproductive capacity, gastrointestinal function, general appearance, or comport. Mathieson, *et al.* (91), reported the same results and further stated after five weeks of similar treatment the offspring of such animals were entirely normal. Hays, *et al.* (96), fed Pyribenzamine, 3 mg./Kg. per day, for six weeks to male and female rats. With a normal caloric intake there was no difference between medicated and nonmedicated animals. With a reduced caloric intake, the controls and medicated animals showed a 50% loss in body weight as compared to the normally fed controls. However, this loss was due to reduced caloric intake and not to the Pyribenzamine. There were no changes in red cell, white cell, or hematocrit values.

Lee, *et al.* (45), found no pathological lesions, grossly or microscopically, after feeding 10 mice 20 mg./Kg. of Thenylene daily for four weeks. Hazel (62) reported that rats receiving Thenylene as 0.05 to 1% of their diets for more than five months showed normal growth and activity.

Dews and Graham (97) gave Neoantergan in doses of 35 mg./Kg. subcutaneously twice daily and observed arrested growth on the seventh day. When the drug was discontinued normal growth resulted. The administration of 16 mg./Kg. daily had no effect upon growth. Graham (94) found that the daily subcutaneous administration of 20 mg./Kg. of Neoantergan, Antistine, or Benadryl had no effect upon the weight gain of weanling mice.

Pogge (98) stated that rats receiving 10 mg./Kg. of Neoantergan daily for six months and up to 200 mg./Kg. daily for thirty-two days showed no signs of chronic toxicity nor was there any gross or microscopic evidence of abnormalities due to the drug. Similar results were seen in dogs receiving 20 mg./Kg. of the drug five times weekly for six months and in monkeys receiving 50 mg./Kg. daily for thirty-five days.

Strauss (51) could find no evidence of toxicity due to Thephorin when fed in a daily dose of 35 mg./Kg. to rats for fifteen weeks. Growth, red cell, white cell, and hemoglobin values were unchanged and the histological examination of the various organs revealed no pathological lesions.

Litchfield, *et al.* (54), fed two male and two female dogs 30 mg./Kg. of Bromothen daily for sixty days. There was no loss in body weight, change in feeding habits, or changes in hematological characteristics. However, salivation occurred in all animals and this became a conditioned reflex in the two males after twenty-five days. No changes were observed in two groups of mice at 20 and 160 mg./Kg. Bromothen fed for thirty days. Similar results were obtained with weanling rats at a dosage level of 65 mg./Kg. but at 700 mg./Kg. the animals had a depressed daily food intake and a 30% less weight gain than the controls. No gross pathology was found in any of the animals.

Anthallan administered to mice, rats, and guinea pigs in doses from 15 to 500 mg./Kg. for periods up to fourteen months had little or no effect upon body weight gain or upon the breeding characteristics or fertility of the animals. No gross or microscopic pathological lesions were found (81).

Feinstone, *et al.* (46), observed no effect upon the weight or hemoglobin values of weanling rats fed 50 mg./Kg. or subcutaneously injected with 20 mg./Kg. of Heteramine daily for thirty-five days.

HISTAMINE BLOCKING ACTION

Effect on Blood Pressure.—Wells, *et al.* (101), reported that the depressor effect of 0.001–0.002 mg./Kg. of histamine on the blood pressure of dogs was abolished by 3 mg./Kg. of Benadryl. After 0.016–0.032 mg./Kg. of histamine this blocking effect was not as marked. They found that a direct proportionality existed between the dose of histamine injected and the amount of this dose antagonized by a given amount of Benadryl. The maximum inhibition (94%) was obtained with 4 mg./Kg. of Benadryl.

In dogs, 1–3 mg./Kg. of Benadryl significantly reduced the depressor response elicited by 4–15 μg. of histamine. These reductions of 58% to 68% showed that Benadryl had an optimum effect at the lower dose (59).

Benadryl has been shown to be equal to Pyribenzamine and Neoantergan in counteracting the depressor effect of 5–10 μg./Kg. of histamine diphosphate (71).

Page and Green (58) reported that Benadryl easily blocked the depressor response of the dog to intravenous histamine. Further, a similar blocking action was observed in the perfused rabbit's ear. However Benadryl did not completely block the response to injected dog or rabbit serum.

Wells, Morris, and Dragstedt (152) found that 10 mg./Kg. of Benadryl significantly reduced the vasodepressor effects of a shocking dose of horse serum in sensitized dogs. It was shown, by the cat blood pressure assay, that 10 μg. of histamine were liberated in one of the test animals but it could not be concluded that histamine was the only vasodepressor agent liberated in shock.

Yonkman, *et al.* (102), found that 3 mg. of Pyribenzamine intravenously in dogs counteracted the hypotensive effect of 15 μg. of histamine, but had no effect upon the hypotension caused by 1 cc. of horse serum intravenously in sensitized animals.

Yonkman, *et al.* (252), showed that Pyribenzamine in doses of 1–3.5 mg./Kg. in sensitized dogs prevented anaphylactic shock due to the injection of antigen. Very slight decreases in blood pressure occurred but the pulse pressure remained relatively constant. Respiration was increased.

Thenylene, 1 mg./Kg. intravenously in cats, abolished the depressor effect of 0.4 μg. of histamine by the same route and significantly reduced the response to larger doses of histamine. An oral dose of 15 mg./Kg. of Thenylene completely abolished the response to 0.5 μg. of injected histamine. Premedication with 5 μg./Kg. of Thenylene intravenously re-

duced the depressor response caused by 0.1–0.3 μg. of histamine by at least 50% (62).

The depressor effect of histamine on the blood pressure of dogs is diminished or annulled by alpha-naphthylmethylethyl-β-chloroethylamine HCl (64).

The vasodilatation, hypotension, and shock caused by 0.25–3 mg./Kg. of intravenous histamine in chloralose or urethane dogs was counteracted by 10 mg./Kg. of 2325 RP or 5 mg./Kg. of Antergan (42).

In the rabbit, 5 mg. of Neoantergan partially reduced the depressor response caused by 0.25 mg. of histamine and abolished entirely the respiratory stimulation usually seen after histamine. In chloralose cats, Neoantergan had no effect on the blood pressure fall due to histamine, but did abolish the action of histamine on the spleen. However, in etherized cats Neoantergan significantly reduced the depressor effect of histamine. Neoantergan also reduced the vasodilator effect of histamine in the dog hind limb preparation. Histamine constriction of the blood vessels of the perfused rabbit's ear was significantly reduced by premedication with 0.001 μg. of Neoantergan and abolished with 0.01 μg. of the drug (97).

Bovet, Horclois, and Fournel (67) observed that premedication with 0.1 mg./Kg. of Neoantergan diminished or abolished the depressor effect of small and moderate doses of histamine in dogs.

When compared by the cat blood pressure method the depressor effect of 2.5 mg./Kg. of histamine was reduced by 50% by 0.14 mg./Kg. Neoantergan, 1.3 mg./Kg. Antistine, or 1.7 mg./Kg. Benadryl (108).

Lehmann and Knoefel (69) showed that diethylaminoethyl-9,10-dihydroanthracene carboxylate, 0.5 μg./Kg., decreased by 50% the depressor effect of 1 and 2 μg./Kg. of histamine on the cat's blood pressure.

Ramanamanjary (113) found that subcutaneous premedication with 5 mg./Kg. of 929 F, 20 mg./Kg. of 1571 F, 1 mg./Kg. Antergan, or 0.5 mg./Kg. Neoantergan prevented the depressor effect of 0.2 mg./Kg. of histamine on the rabbit blood pressure. Antergan and Neoantergan were not effective when chloralose, barbiturates, or urethane was the anesthetic.

Minard and Rosenthal (80) reported that thymoxyethyldiethylamine, 40 mg./Kg. subcutaneously reduced the degree and duration of hemoconcentration but not the vasodepressor effect of 1 mg./Kg. of histamine.

Marsh and Davis (49) confirmed previous reports that thymoxyethyldiethylamine, 1571 F, Benadryl, and Antergan counteracted the vasodepressor effects of intravenous histamine in dogs. In equal doses 4.4 mg./Kg. the drugs rated as follows: Benadryl 125, Antergan 80, Pyribenzamine 40, thymoxyethyldiethylamine 12, and 1571 F 8. Similar ratings were obtained at higher doses of Benadryl and Antergan. All the compounds antagonized the effect of histamine on the rabbit blood pressure response and no differences in potency could be determined. The drugs also antagonized the vasodepressor effects of histamine in rats and guinea pigs.

Dreyer and Harwood (82) reported that Neoheteramine (2–5 mg./Kg.) counteracted the vasodepressor effect of 1–2 μg. of histamine, but larger doses of histamine, 5–7 μg./Kg., could not be offset by 8–10 mg./Kg. of the former. However, the vasoconstrictor effect of histamine on the perfused rabbit's ear was counteracted by Neoheteramine.

Lehmann, *et al.* (75), reported that Thephorin either abolished or prevented the vasodilator effect of histamine in normal cats. In decapitated cats the vasopressor response to histamine was blocked. Thephorin reversed the blood pressure effect seen when histamine was injected into rabbits.

Halpern (131) found that 3277 RP completely blocked the vascular effects of 0.1–1 mg./Kg. of histamine in the urethanized rabbit and the hypotensive effects in the dog and cat.

Experimental Anaphylaxis.—Loew and Kaiser (122) passively sensitized guinea pigs with rabbit antiovalbumin serum, then injected intrajugularly 2 cc. of a 2% solution of crystalline ovalbumin twenty-four hours later. Three milligrams of Benadryl protected 92.9%, 3 mg. of 1571 F protected 85%, and 12 mg. of β-morpholinoethyl benzhydryl ether protected 100% of the animals. Selle (123) confirmed the protective action of Benadryl by his work with egg albumin shock.

Mayer, *et al.* (149), found that although 2% aerosol of Pyribenzamine protected sensitized guinea pigs against the immediate toxic effects of a shocking dose of antigen, many of the animals died within twenty-four hours. This toxic effect was shown to be due to the antigen because there was no evidence of lung damage in animals treated with the drug alone.

Mayer and Brousseau (151) reported that Pyribenzamine in doses of 10–50 mg./Kg. protected sensitized mice from the lethal effects of a shocking dose of antigen.

Arbesman, *et al.* (227), found that 1, 1.5, and 3 mg./Kg. of Pyribenzamine intraperitoneally in sensitized guinea pigs protected the majority of the animals against anaphylactic shock produced by the intracardial injection of 1 cc. of human serum. The protective effect was greater with larger doses.

Marcus (135) reported that premedication with 5–10 mg. of Pyribenzamine or Benadryl ten to thirty minutes before a shocking dose of antigen protected guinea pigs against 8 lethal doses of antigen. Similar results were obtained by Mayer (142) with lower doses (2.5–5 mg./Kg.) of Pyribenzamine.

Achenbach and Loew (64) observed that the subcutaneous injection of 0.025 to 0.1 mg./Kg. of alpha - naphthylmethylethyl - β - chloroethylamine HCl prevented anaphylactic shock in guinea pigs.

Vallery-Radot, *et al.* (117), showed that Antergan at a dose of 10–20 mg./Kg. prevented serum shock in rabbits. However, this dose is much larger than that which protects guinea pigs and dogs. The intramuscular injection of 40 mg./Kg. of Antergan in rabbits prevented anaphylaxis from the injection of horse serum. However, if the antigen was again injected eighteen to thirty-two hours later anaphylactic shock occurred. This suggests that antibody or antigen neutralization occurs only as long as the antihistamine is in the body and that there is no permanent neutralization (118).

Halpern (42) protected sensitized guinea pigs against a shock dose of cattle serum with 25–50 mg./Kg. of 2325 RP or 20 mg./Kg. of Antergan. Both compounds were able to prevent anaphylactic spasm of the sensitized guinea pig ileum *in vitro*. In horse serum sensitized dogs, 10 mg./Kg. of 2325 RP or 5 mg./Kg. Antergan prevented the depressor effect of 1 cc. of horse serum. The same protective

action was observed when Witte Peptone was used instead of horse serum.

Rose, *et al.* (145), showed that when equal, large doses (3 mg./Kg.) were administered to sensitized guinea pigs Neoantergan, Pyribenzamine, Antergan, and Benadryl were equivalent in protective power. However, when the doses were 0.1 mg./Kg. the drugs were rated as follows: Pyribenzamine, Antergan, Neoantergan, and Benadryl. The latter two compounds were equally effective in counteracting the antigen-induced spasm of the sensitized guinea-pig ileum. These results do not confirm the findings of Friedlaender, *et al.* (146), in so far as the lower dose is concerned but do confirm the equivalent potency of Pyribenzamine and Benadryl at the 3 mg./Kg. dose.

Lehmann and Young (44) compared equal doses (50 mg./Kg.) of diethylaminoethyl-9,10-dihydroanthracene carboxylate, diethylaminoethyl-xanthene carboxylate and diethylaminofluorene-9-carboxylate in a large series of sensitized guinea pigs and found that the first compound gave 100% protection, the second 70%, and the last only 37%.

Rosenthal and Brown (78) showed that 0.5 mg. of thymoxyethyldiethylamine prevented the sensitized guinea-pig ileum from responding to the antigen *in vitro*. Similar results were obtained on the isolated guinea-pig uterus. *In vivo* experiments on guinea pigs showed that although all animals were protected against a dose of 0.04 mg./30 Gm. of antigen, larger doses caused a fatal reaction.

Staub and Bovet (105) have shown that 929 F protects guinea pigs from serum-induced anaphylactic shock. However, it does not protect sensitized rabbits against anaphylactic serum shock (115).

Reinhard and Scudi (133) found that 10 mg./Kg. intraperitoneally of both Neoheteramine and Pyribenzamine protected 60% of the sensitized guinea pigs against a shocking dose of horse serum. Benadryl protected only 30% at a dose of 25 mg./Kg.

Intraperitoneal injection of 2 mg./Kg. of Thephorin in sensitized guinea pigs protected 58% of the animals from fatal anaphylactic shock caused by the intracardial injection of 0.5 cc. of a 2% egg white solution (75).

Vallery-Radot, *et al.* (104), reported that 933 F and 883 F sometimes protected rabbits against anaphylactic shock. Rapid injection of 883 F prevents anaphylactic shock in rabbits (109).

Halpern (131) found that when sensitized guinea pigs received a shocking dose of serum 80% were protected by 0.1 mg./Kg. of 3277 RP, 60% by 2 mg./Kg. of 3015 RP, 67% by 0.5 mg./Kg. of Neoantergan, and 100% by 0.5 mg./Kg. of Antergan. Thus it would appear that the phenothiazine derivatives 3277 RP and 3015 RP are as potent as the older compounds in prevention of the fatal outcome due to anaphylactic shock.

Fatal anaphylactic effects caused by the intracardial injection of 1 cc. of horse serum in sensitized guinea pigs were prevented by protection with 3.5 mg./Kg. intraperitoneally of Heteramine (46).

Fifty mg./Kg. of 1135 F (*p*-hydroxyphenoxyethyldiethylamine) frequently but not always protected rabbits against fatal anaphylactic serum shock (129).

Experimental Skin Sensitization.—Dreisbach (99, 100) reported that neither Benadryl nor Pyribenzamine, 5 mg./Kg. subcutaneously twice daily, prevented the Arthus phenomenon in rabbits sensitized to penicillin or horse serum. Further, when these drugs were mixed with penicillin or horse serum they did not prevent a typical allergic response. However, when they were mixed with histamine under the same experimental conditions they did prevent the typical histamine wheal.

Benadryl does not sensitize or produce anaphylactic reactions in guinea pigs (50).

Mayer and Kull (134) found that pretreatment with Pyribenzamine almost completely suppressed the response of paraphenylenediamine-sensitized guinea pigs regardless of whether the antigen was injected with or without hyaluronidase.

Fischel (132) observed no modification in Arthus phenomenon induced in rabbits by the anti-egg albumin serum when Pyribenzamine was administered.

Mayer (142) showed that Pyribenzamine in doses of 10–25 mg./Kg. was effective in counteracting the urticarial action of hog serum in sensitized guinea pigs. However, after the effects of the drug had worn off the allergic response returned. In guinea pigs sensitized to paraphenylenediamine, Pyribenzamine and Antergan prevented the dermatitis seen in the unmedicated controls. Pyribenzamine was also effective in counteracting contact dermatitis in guinea pigs due to 2,4-dinitrochlorobenzene. Further work has shown that Pyribenzamine is effective in reducing the time of onset of the dermatitis caused by the local application of 10% mustard oil to guinea pigs (143).

Rennick, *et al.* (158), showed that wheal reaction to intradermal histamine in rabbits could be prevented by 1 mg./Kg. of Pyribenzamine intravenously or by 300 mg./Kg. orally. Further, capillary permeability as determined by the dye method is not increased.

Cullumbine (251) reported that Neoantergan subcutaneously, 10–40 mg./Kg. thirty minutes prior either to burning or injection of histamine or leucotaxine, prevented the spread of injected dye due to histamine but not to burning or leucotaxine.

Mixtures of Thephorin 1% and histamine 1:1000 to 1:10,000 when injected into the skin of the rabbit prevented the usual wheal formation and the spread of Trypan Blue seen when histamine alone was injected (51).

In the prevention of the spread of Trypan Blue in rabbits injected intracutaneously with histamine, the drugs used were rated as follows: Pyribenzamine, Neoheteramine, and Benadryl. Pyribenzamine was about five times more potent than either of the other drugs (137).

Last and Loew (144) showed that both Benadryl and Neoantergan reduced the spread of Trypan Blue usually seen after intradermal injections of histamine. Both drugs were effective when trypsin, tetracaine, and heparin were used instead of histamine but the responses were not significantly different from those of the controls. Benadryl produced no qualitative or quantitative differences in the spread of the dye when $MgSO_4$, horse serum, cobra venom, insulin, or staphylococcus toxin was injected.

Diethylaminoethyl - 9,10 - dihydroanthracene carboxylate intensified the intradermal reaction to histamine in rabbits when the drugs were injected together (44).

Boquet (108) found that 929 F, 883 F, and Anter-

gan had no effect on the intradermal reaction to tuberculin.

Respiratory Tract Effects.—Ellis and Newsome (124) tested the potency of a series of antihistaminic compounds related to Benadryl and found that tertiary amine derivatives such as Benadryl were more effective in counteracting histamine-induced bronchio-constriction in the perfused guinea-pig lung than quaternary ammonium derivatives. In the dog lung preparation *in situ* Benadryl was the most potent of all the compounds tested. The drugs were given in doses of 3 to 6 mg./Kg. after maximum constriction had developed due to the administration of 0.1 to 0.3 mg./Kg. of histamine. This confirms the previous observations of Ellis (156). However, Loew *et al.* (157) reported that conversion of Benadryl to a quaternary derivative increased its potency against the histamine bronchio-constriction.

Yonkman, *et al.* (102), found that Pyribenzamine, 25–50 μg., reduced bronchio-spasm induced by 50 μg. of histamine in the isolated guinea-pig lung. There was no marked effect on normal bronchial tonus. Depending upon the dose the relaxant effect lasted twenty to forty minutes and if Pyribenzamine was injected at the time of maximum constriction the protective effect of the drug was prolonged. In a similar series of experiments Antergan was as effective as Pyribenzamine against histamine spasm, but against the spasm caused by horse serum in sensitized lungs Pyribenzamine was not very effective. Pyribenzamine, 2 mg. intravenously, counteracted the spasmogenic effect of 15–50 μg. of histamine on the dog lung *in vivo*. The action was of long duration—three hours or more. Inasmuch as sensitized dogs did not exhibit a bronchial spasm upon injection of horse serum, Pyribenzamine could not be shown to be effective against this aspect of anaphylaxis.

Mayer, *et al.* (90), reported that 50 μg. of Pyribenzamine counteracted the bronchio-spasm induced by 50 μg. of histamine. This effect on the isolated guinea-pig lung was of forty to ninety minutes' duration.

Rennick, *et al.* (158), showed that 25–50 μg. of Pyribenzamine prevented bronchio-constriction usually produced by 50 μg. of histamine in the perfused guinea-pig lung. However, Pyribenzamine did not prevent bronchio-constriction in the horse serum sensitized lung upon addition of the antigen to the perfusion fluid.

Bronchio-spasm induced in guinea pigs by the intravenous injection of 1 mg./Kg. of histamine was prevented by the injection of 1 mg./Kg. of Antergan (42).

Sherrod, *et al.* (71), compared Neoantergan, Pyribenzamine, and Benadryl and found that the first two compounds were more potent in combating experimental bronchio-spasm in guinea pigs.

Lehmann and Young (44) found diethylaminoethyl-9,10-dihydroanthracene carboxylate in a dose of 1 mg. effectively produced bronchio-dilatation in the sensitized guinea-pig lung *in vitro*.

Lehmann, *et al.* (75), reported that 1 mg./Kg. of Thephorin prevented histamine-induced bronchio-spasm in cats.

929 F and 1571 F prevent the excitation provoked by histamine on the bronchioles (103).

Bronchio-constriction in the guinea pig due to histamine was prevented by premedication with 5 mg./Kg. of 929 F, 20 mg./Kg. of 1571 F, 1 mg./Kg. Antergan, or 0.5 mg./Kg. Neoantergan (113).

Injected Histamine.—Selle (123) stated that 10 mg. of Benadryl protected guinea pigs against 3 *MLD* of histamine.

Wells and Morris (155) have stated that the amount of injected histamine inhibited bears a constant relationship to the amount of Benadryl injected within certain limits (1–16 mg./Kg.). Histamine is blocked at its site of action.

Against intravenous histamine, Pyribenzamine was more potent than Benadryl, Neoheteramine, or Heteramine. The second and third compounds were almost equivalent in potency (133).

Mayer, *et al.* (149), reported that when guinea pigs inhaled a 2% aerosol of Pyribenzamine they were protected against 7–15 *MLD* of intracardially injected histamine. Further, the duration of protection was relatively long.

Mayer and Brousseau (151) found that, even in doses of 25 mg./Kg. of Pyribenzamine or Benadryl, mice were not protected from the lethal effects of injected histamine; in fact, the toxic action of the latter was potentiated by both drugs.

Intraperitoneal or subcutaneous injection of 1 mg./Kg. of Thenylene protected guinea pigs against the shock induced by 0.4 mg./Kg. (LD_{100}) of histamine and 5 mg./Kg. of the antihistamine protected against 24 mg./Kg. of histamine (62).

Subcutaneous or intraperitoneal injection of 0.025 to 0.1 mg./Kg. of alpha-naphthylmethylethyl-β-chloromethylamine prevents or annuls the symptoms of shock in the guinea pig due to injected histamine. This antagonism is rapid in onset and markedly prolonged (64).

Halpern (42) found that 2325 RP in doses of 40 mg. protected guinea pigs against 50 fatal doses of histamine, whereas 10 mg. of Antergan counteracted 60 fatal doses of histamine.

Bovet, Horclois, and Walthert (43) reported that Neoantergan, 1 mg./Kg. subcutaneously, protected guinea pigs against 45 mg. of histamine intravenously (LD_{75}).

Lehmann and Knoefel (68) reported diethylaminoethyl-9,10-dihydroanthracene carboxylate (50 mg./Kg. subcutaneously) protected guinea pigs against 1.7 mg./Kg. of histamine phosphate intracardially.

Rosenthal and Brown (78) showed that thymoxyethyldiethylamine, 40 mg./Kg. subcutaneously, protected guinea pigs injected intracardially with 0.5 mg./Kg. of histamine.

Rose, *et al.* (145), rated the antihistamine drugs on their ability to protect guinea pigs from fatal histamine shock as follows: Neoantergan 125, Pyribenzamine 37, Antergan 6, and Benadryl 5. The numbers signified the number of LD_{100} of histamine counteracted by 3 mg./Kg. of antihistamine drug. Further, in equal doses Neoantergan was 4 times more potent than Benadryl. Friedlaender, *et al.* (146), rated the drugs as follows: Neoantergan 3.5 times more active than Pyribenzamine which is 6 to 7 times more active than either Antergan or Benadryl. This is in accord with their previous findings in which Pyribenzamine was found to be much more potent than Benadryl (147).

Bukantz and Dammin (159), using a method for measuring the fluorescence in the skin of rabbits, dogs, and humans after intravenous fluorescein, showed that intracutaneous histamine (1:10,000) could be blocked by Benadryl or Neoheteramine in a

concentration of 1:10,000 only in the last two species.

Thephorin, 5 mg./Kg. intraperitoneally, protected guinea pigs against 10–12 intracardial fatal doses of histamine and against death from anaphylactic shock (75).

Bromothen and Chlorothen were both three times more potent than Pyribenzamine in counteracting intravenous histamine in guinea pigs and, compared with Thenylene, Bromothen was twice as potent (54).

In the guinea pig, Halpern (131) found that 3277 RP counteracted 1500–1600 lethal doses of histamine while 3015 RP counteracted 400, Neoantergan 80, Antergan 50 to 60, and thymoxyethyldiethylamine 2 to 3. In rabbits 3277 RP counteracted 400–450 lethal doses of histamine, 3015 RP counteracted 100–125, Neoantergan 30, and Antergan 20–25. 3277 RP was not tested in the dog but the 3015 RP counteracted 2–25 lethal doses of histamine.

Staub (103) found that 929 F and 1571 F prevented histamine shock in guinea pigs. Staub and Bovet (105) had observed this effect with 929 F in 1937. Further, Vallery-Radot, *et al.* (104); observed this protective action in rabbits when 933 F and 883 F were administered. They also reported that rapid injection of 883 F immediately before the shocking dose prevents anaphylaxis (109). Vallery-Radot, Mauric, and Holtzer (115) found that 929 F strongly antagonized histamine shock in rabbits.

Premedication with 4, 5, and 6 mg./Kg. of Heteramine protected guinea pigs against the fatal histamine shock produced by the intracardial injection of 0.85 mg/Kg. of the latter (46).

Gastric Secretion.—Loew, *et al.* (59), found that Benadryl in a dose of 10 mg./Kg. subcutaneously to Heidenheim pouch dogs reduced gastric secretion in 3 of the 4 histamine-stimulated animals by 40%. One animal showed no reduction in secretion.

Sangster, Grossman, and Ivy (138) found that neither Benadryl nor Pyribenzamine (50 mg. subcutaneously) reduced the volume of secretion or the free acid output in four pouch dogs injected with histamine.

Friesen, Baronofsky, and Wangensteen (154) found that Benadryl had no effect upon the gastric secretory response of pouch dogs injected with histamine. Further, 100 mg. of Benadryl in beeswax mixture intramuscularly failed to protect dogs from histamine provoked ulcer.

Reuse (139) showed that Antergan, Neoantergan, 3277 RP, and Benadryl had no effect upon histamine induced gastric secretion in the guinea pig and cat.

Graham (94) reported that Neoantergan, Benadryl, or Antistine in doses of 4 mg./Kg. cutaneously in guinea pigs did not reduce the combined acid or, abolish the free acid content of the ligated stomach. However, Neoantergan was the most potent in reducing the quantity of free acid induced by histamine.

Lehmann and Young (44), using dogs, showed that 20 mg./Kg. of diethylaminoethyl-9,10-dihydroanthracene carboxylate subcutaneously reduced the volume but not the acidity of histamine-induced gastric secretion. Lehmann and Knoefel (110) found that diethylaminoethylfluorene-9-carboxylate had a similar effect on meat extract stimulated gastric secretion. Both compounds were equal in potency in counteracting gastric motility induced by injection of 0.5 unit/Kg. of insulin in dogs.

Bourque and Loew (112) found that 1571 F had no effect upon food-stimulated gastric secretion in pouch dogs and pretreatment with 929 F did not modify gastric secretion in dogs injected with pilocarpine. Burchell and Varco (127) reported that thymoxyethyldiethylamine had no effect upon histamine-stimulated gastric secretion in Heidenheim pouch dogs.

Dreyer and Harwood (82) showed that 1–5 mg./Kg. of Neoheteramine did not reduce the total or free acid secretion in the cat when the left vagus was stimulated.

Halpern (131) was unable to show that the antihistamine drugs of the phenothiazine series (3015 RP, 3277 RP, etc.) had any effect upon gastric secretion in experimental animals. Further, these drugs although they were able to counteract histamine shock were unable to prevent histamine-induced ulceration and perforation of the stomach. This confirmed Halpern's previous observation (137) that neither 3015 RP nor 3277 RP prevented fatal histamine-induced stomach ulcer in guinea pigs although the other effects of the latter agent were blocked.

Histamine Asthma.—Intraperitoneal injection of 1.8 ±0.3 mg./Kg. of Benadryl protected 50% of the guinea pigs exposed to histamine aerosol (101)

Loew, Kaiser, and Moore (153) compared many drugs for their effect on histamine asthma in guinea pigs and rated them as follows: epinephrine, Benadryl, Demerol, atropine, papaverine, and aminophylline. Antispasmodics such as Trasentin, Pavatrine, and Syntropan which counteract acetylcholine were ineffective against histamine as were the local anesthetics, procaine and Nupercaine. Ergotamine tartrate likewise had no effect and physostigmine and ephedrine increased the severity of the asthma. It was concluded that a careful selection of drugs is necessary in order to obtain selective antispasmodic action on various smooth muscles.

Reinhard and Scudi (133) compared several antihistamines for their protective action against histamine asthma and rated them as follows: Pyribenzamine, Neoheteramine and Benadryl, and Heteramine. Further, when vaporized simultaneously with histamine, Pyribenzamine had more than four times the potency of Neoheteramine.

Lee, *et al.* (45), reported that the median protective dose of Thenylene was 0.152 ± 0.023 mg./Kg. as compared to Benadryl, 1.27 ± 0.36 mg./Kg. and Pyribenzamine, 0.059 ± 0.015 mg./Kg. when tested on guinea pigs in a histamine atmosphere. The concentration of histamine acid phosphate used was 0.187 μg./liter of air at a rate of 9 liters per minute.

Feinberg and Bernstein (194) found Thenylene more effective than Benadryl in counteracting histamine asthma in guinea pigs. However, Pyribenzamine was more potent than Thenylene under the same conditions.

Halpern (42) protected guinea pigs from the fatal effect of breathing atomized histamine for thirty minutes with 20 mg./Kg. of 1571 F, 10 mg./Kg. of 2325 RP, and 1.0 mg./Kg. of Antergan. Larger doses of the last two compounds protected animals for longer periods of time.

Neoantergan was four times more potent than Antistine and twice as potent as Benadryl when tested with histamine asthma in guinea pigs, and Benadryl was twice as potent as Antistine under the same conditions (94).

gan had no effect on the intradermal reaction to tuberculin.

Respiratory Tract Effects.—Ellis and Newsome (124) tested the potency of a series of antihistaminic compounds related to Benadryl and found that tertiary amine derivatives such as Benadryl were more effective in counteracting histamine-induced bronchio-constriction in the perfused guinea-pig lung than quaternary ammonium derivatives. In the dog lung preparation *in situ* Benadryl was the most potent of all the compounds tested. The drugs were given in doses of 3 to 6 mg./Kg. after maximum constriction had developed due to the administration of 0.1 to 0.3 mg./Kg. of histamine. This confirms the previous observations of Ellis (156). However, Loew *et al.* (157) reported that conversion of Benadryl to a quaternary derivative increased its potency against the histamine bronchio-constriction.

Yonkman, *et al.* (102), found that Pyribenzamine, 25–50 μg., reduced bronchio-spasm induced by 50 μg. of histamine in the isolated guinea-pig lung. There was no marked effect on normal bronchial tonus. Depending upon the dose the relaxant effect lasted twenty to forty minutes and if Pyribenzamine was injected at the time of maximum constriction the protective effect of the drug was prolonged. In a similar series of experiments Antergan was as effective as Pyribenzamine against histamine spasm, but against the spasm caused by horse serum in sensitized lungs Pyribenzamine was not very effective. Pyribenzamine, 2 mg. intravenously, counteracted the spasmogenic effect of 15–50 μg. of histamine on the dog lung *in vivo*. The action was of long duration—three hours or more. Inasmuch as sensitized dogs did not exhibit a bronchial spasm upon injection of horse serum, Pyribenzamine could not be shown to be effective against this aspect of anaphylaxis.

Mayer, *et al.* (90), reported that 50 μg. of Pyribenzamine counteracted the bronchio-spasm induced by 50 μg. of histamine. This effect on the isolated guinea-pig lung was of forty to ninety minutes' duration.

Rennick, *et al.* (158), showed that 25–50 μg. of Pyribenzamine prevented bronchio-constriction usually produced by 50 μg. of histamine in the perfused guinea-pig lung. However, Pyribenzamine did not prevent bronchio-constriction in the horse serum sensitized lung upon addition of the antigen to the perfusion fluid.

Bronchio-spasm induced in guinea pigs by the intravenous injection of 1 mg./Kg. of histamine was prevented by the injection of 1 mg./Kg. of Antergan (42).

Sherrod, *et al.* (71), compared Neoantergan, Pyribenzamine, and Benadryl and found that the first two compounds were more potent in combating experimental bronchio-spasm in guinea pigs.

Lehmann and Young (44) found diethylaminoethyl-9,10-dihydroanthracene carboxylate in a dose of 1 mg. effectively produced bronchio-dilatation in the sensitized guinea-pig lung *in vitro*.

Lehmann, *et al.* (75), reported that 1 mg./Kg. of Thephorin prevented histamine-induced bronchio-spasm in cats.

929 F and 1571 F prevent the excitation provoked by histamine on the bronchioles (103).

Bronchio-constriction in the guinea pig due to histamine was prevented by premedication with 5 mg./Kg. of 929 F, 20 mg./Kg. of 1571 F, 1 mg./Kg. Antergan, or 0.5 mg./Kg. Neoantergan (113).

Injected Histamine.—Selle (123) stated that 10 mg. of Benadryl protected guinea pigs against 3 *MLD* of histamine.

Wells and Morris (155) have stated that the amount of injected histamine inhibited bears a constant relationship to the amount of Benadryl injected within certain limits (1–16 mg./Kg.). Histamine is blocked at its site of action.

Against intravenous histamine, Pyribenzamine was more potent than Benadryl, Neoheteramine, or Heteramine. The second and third compounds were almost equivalent in potency (133).

Mayer, *et al.* (149), reported that when guinea pigs inhaled a 2% aerosol of Pyribenzamine they were protected against 7–15 *MLD* of intracardially injected histamine. Further, the duration of protection was relatively long.

Mayer and Brousseau (151) found that, even in doses of 25 mg./Kg. of Pyribenzamine or Benadryl, mice were not protected from the lethal effects of injected histamine; in fact, the toxic action of the latter was potentiated by both drugs.

Intraperitoneal or subcutaneous injection of 1 mg./Kg. of Thenylene protected guinea pigs against the shock induced by 0.4 mg./Kg. (LD_{100}) of histamine and 5 mg./Kg. of the antihistamine protected against 24 mg./Kg. of histamine (62).

Subcutaneous or intraperitoneal injection of 0.025 to 0.1 mg./Kg. of alpha-naphthylmethylethyl-β-chloromethylamine prevents or annuls the symptoms of shock in the guinea pig due to injected histamine. This antagonism is rapid in onset and markedly prolonged (64).

Halpern (42) found that 2325 RP in doses of 40 mg. protected guinea pigs against 50 fatal doses of histamine, whereas 10 mg. of Antergan counteracted 60 fatal doses of histamine.

Bovet, Horclois, and Walthert (43) reported that Neoantergan, 1 mg./Kg. subcutaneously, protected guinea pigs against 45 mg. of histamine intravenously (LD_{75}).

Lehmann and Knoefel (68) reported diethylaminoethyl-9,10-dihydroanthracene carboxylate (50 mg./Kg. subcutaneously) protected guinea pigs against 1.7 mg./Kg. of histamine phosphate intracardially.

Rosenthal and Brown (78) showed that thymoxyethyldiethylamine, 40 mg./Kg. subcutaneously, protected guinea pigs injected intracardially with 0.5 mg./Kg. of histamine.

Rose, *et al.* (145), rated the antihistamine drugs on their ability to protect guinea pigs from fatal histamine shock as follows: Neoantergan 125, Pyribenzamine 37, Antergan 6, and Benadryl 5. The numbers signified the number of LD_{100} of histamine counteracted by 3 mg./Kg. of antihistamine drug. Further, in equal doses Neoantergan was 4 times more potent than Benadryl. Friedlaender, *et al.* (146), rated the drugs as follows: Neoantergan 3.5 times more active than Pyribenzamine which is 6 to 7 times more active than either Antergan or Benadryl. This is in accord with their previous findings in which Pyribenzamine was found to be much more potent than Benadryl (147).

Bukantz and Dammin (159), using a method for measuring the fluorescence in the skin of rabbits, dogs, and humans after intravenous fluorescein, showed that intracutaneous histamine (1:10,000) could be blocked by Benadryl or Neoheteramine in a

capillaries so that capillary dilatation due to histamine occurs even after Benadryl.

Neoantergan, 25 μg., reduced the dilatating effect of 25 μg. of histamine on the perfused cat heart coronary vessels and on the coronary vessels of the dog heart-lung preparation. Further, the drug abolished the stimulant effect of histamine on the isolated rabbit auricles. Upon the driven rabbit auricles, Neoantergan was twice as active as quinidine in reducing the rate of contraction (97).

Reinhard and Scudi (133) found that Neoheteramine and Pyribenzamine were equal in combating histamine spasm of the excised guinea-pig tracheal chain. Castillo and de Beer (76, 77) found Benadryl also counteracted the histamine spasm of the tracheal chain.

Effect on the Isolated Intestine.—The spasmogenic effect of histamine on the intact dog duodenum was counteracted by Benadryl, Pyribenzamine, and Neoantergan (71).

Winder, *et al.* (87), found that 1.1 mg. of Benadryl/100 cc. of solution reduced the spasm elicited by 8 μg. of histamine by 68%.

Selle (123) found that 0.1 mg. of Benadryl counteracted the spasmogenic effect of 10 μg. of histamine on the guinea-pig ileum. Upon the sensitized guinea-pig ileum 0.2 mg. of Benadryl was required to counteract the assault dose of antigen.

Pyribenzamine, 0.01–0.02 μg., usually counteracted the spasm produced in the isolated guinea-pig jejunum by 1 μg. of histamine (102).

Hoekstra and Steggerda (72) showed that a dose of 0.5 mg./Kg. of Pyribenzamine did not counteract the effect of 10–30 μg. of histamine on the dog colon *in situ*. However, 1.5–2.5 mg. of the antihistamine was effective in counteracting histamine and the duration of activity was up to fifty minutes.

Cameron and Craver (126), using dogs with Thiry-Vella loops, found that after the administration of antihistaminic drugs, histamine often gave a significant inhibition of motility which might or might not be followed by a secondary stimulation. This action was probably due to stimulation of the sympathetic ganglia by histamine because adrenalectomized animals showed that epinephrine was not the causative agent.

Lee, *et al.* (45), reported that Thenylene, weight for weight, was 4.9 times as active as Benadryl and 0.8 times as active as Pyribenzamine when tested by its relaxant effect on histamine-induced spasm of the isolated guinea-pig ileum. The spasm of the isolated guinea-pig ileum caused by 0.005 μg./cc. of histamine can be inhibited at least 75% or relaxed by 0.002 μg./cc. of Thenylene (62).

Halpern and Mauric (107) found that there was a definite quantitative relationship between the amount of histamine which was antagonized by Antergan when tested with the isolated guinea-pig ileum.

Halpern (42) found that the spasm of guinea-pig intestine induced by histamine could be abolished by 15 μg. of 1571 F, 1.5 μg. of 2325 RP, and 0.1 μg. of Antergan.

Reuse (140) showed that Antergan was effective in preventing serum-induced contraction of the sensitized guinea-pig ileum *in vitro*. In his later work, Reuse (141) found that Antergan, Neoantergan, and 3277 RP all were able to counteract the intestinal spasm of the isolated guinea-pig ileum produced by the H-substance liberated during anaphylactic shock.

Neoantergan (0.1 μg.) reduced the response of the isolated guinea-pig ileum to 1 μg. of histamine by more than half its previous level (97).

Graham (94) found that Neoantergan, Benadryl, and Antistine were rated in that order when tested upon the isolated guinea-pig ileum.

Reuse (130) found that Neoantergan and 3277 RP were much stronger than Antistine in preventing histamine-induced spasm of the guinea-pig ileum *in vitro*. It was also shown that the duration of action and the amount of inhibition observed were proportional to the dose of the drug. Halpern (131) reported that 3277 RP, 3015 RP, and Antergan were equal in antagonizing the effect of histamine on the isolated guinea-pig ileum.

Using a comparative method, Schild (136) found Neoantergan more potent than Benadryl in counteracting histamine spasm of the guinea-pig ileum *in vitro*. This antihistamine effect of Neoantergan was greater the longer the drug remained in contact with the tissue.

Lehmann and Young (44) found diethylaminoethyl-9,10-dihydroanthracene carboxylate 3 times stronger than 1571 F when tested upon the isolated guinea-pig ileum. Lehmann and Knoefel (68, 69) showed that diethylaminoethyl-fluorene-9-carboxylate and diethylaminoethyl-xanthene-9-carboxylate were both less potent than the anthracene compound.

Thymoxyethyldiethylamine (929 F) strongly inhibited histamine-induced intestinal spasm (127).

Rosenthal and Brown (78) found that 0.5 μg. of thymoxyethyldiethylamine prevented the intestinal spasm usually produced by 0.02–0.04 μg. of histamine on the isolated guinea-pig ileum.

Although Neoheteramine was active in abolishing histamine-induced spasm of the isolated guinea-pig ileum it was not so effective on this as on other smooth muscles (82).

Lehmann, *et al.* (75), found that 0.01 mg./cc. of Thephorin blocked the effect of 0.2 mg. of histamine phosphate. The effect increased as the interval between the administration of the two drugs increased.

Chlorothen and Bromothen are equivalent in potency and 4 times more active than Pyribenzamine in suppressing histamine-induced spasm of the guinea-pig ileum. Further, Bromothen is more than 3 times as active as Thenylene by the same test (54).

929 F and 1571 F are antagonistic to the intestinal spasm induced by histamine (103).

Ungar, Parrot, and Bovet (106) antagonized the spasmogenic effect of histamine with 933 F.

Heteramine, 1 μg./cc. of bathing fluid, prevented the contraction of the isolated guinea-pig ileum usually produced by 1 μg./cc. histamine (46).

Effect on the Uterus.—Selle (123) reported that the effect of 10 μg. of histamine on the isolated guinea pig uterus was prevented by 0.1 mg. of Benadryl.

Sherrod, *et al.* (71), found that Benadryl, Pyribenzamine, and Neoantergan all given intravenously in a 3 mg./Kg. dose counteracted the spasmogenic effect of injected histamine on the dog uterus.

Contraction of the isolated guinea-pig uterus produced by 10–20 μg. of histamine was counteracted by 10–50 μg. of Pyribenzamine (90).

Gayet-Hallion and Quivy (116) reported that

Wilcox and Siegal (128) found that 1571 F protected guinea pigs from fatal histamine and anaphylactic shock.

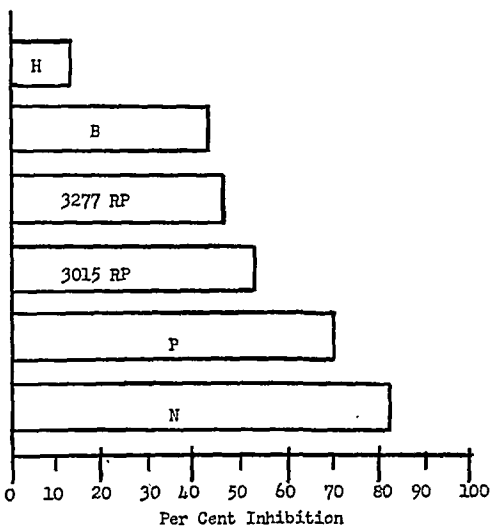
Lehmann, *et al.* (75), found that 0.5 mg./Kg. of Theophorin intraperitoneally protected 50% of the guinea pigs exposed to 0.4 mg. of histamine base per liter of air, while only 2% of the controls survived.

Litchfield, *et al.* (54), found both Chlorothen and Bromothen intraperitoneally three times more potent than Pyribenzamine in prevention of fatal histamine asthma in guinea pigs. Orally Bromothen was the most potent and when compared with Thenylene, Bromothen was about 3 times more potent.

Halpern (131) reported that 3277 RP was twice as effective in counteracting histamine asthma as Antergan and that 3015 RP was equivalent to the latter compound. Further, this action of 3277 RP had a duration of eight to nine hours, while that of Antergan and Neoantergan lasted only three to four hours. All the drugs were given at a dose of 1 mg./Kg.

Feinstone, Williams, and Rubin (46) injected a dose of 0.3 to 2.5 mg./Kg. of Heteramine interperitoneally in guinea pigs and found that the drug significantly and increasingly reduced the mortality caused by breathing the vapor of 5 cc. of a 0.125% histamine solution.

Winter (160) made a comparison of the potency of six antihistamines and his results are given in Graphs I and II.



Graph I.—Inhibition of histamine-induced intestinal contraction in the guinea pig. Equivalent doses of each drug were used.

H—Heteramine.
B—Benadryl.
P—Pyribenzamine.
N—Neoantergan.

Miscellaneous.—Kyser, McCarter, and Stengle (125) found that the Benadryl, 10 mg./Kg. subcutaneously twice daily, was effective in preventing serum-induced myocarditis in rabbits. When the dose was decreased to 2 mg./Kg. the drug was only partially effective.

Traub, *et al.* (148), reported that 7.5 mg./Kg. of Benadryl suppressed the action of 1:10,000 histamine on the skin capillaries of the rabbits by 75% to 100%.

Arbesman, *et al.* (227), found that Pyribenzamine had no effect on the precipitin titer or guinea-pig complement titer.

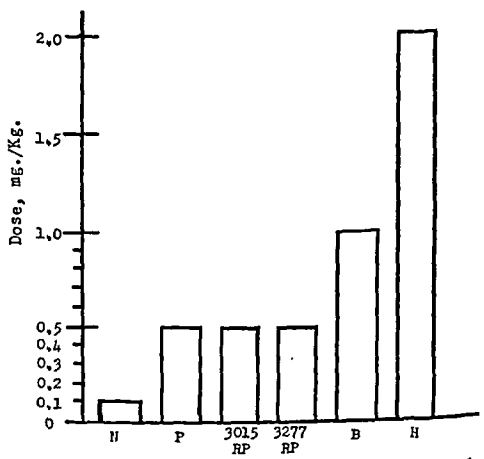
Mayer and Kull (134) showed that Pyribenzamine and Antistine in equal doses had an equivalent effect in the prevention of the spread of India ink injected intradermally in rats which had been injected with hyaluronidase.

Mayer, Eisman, and Aronson (150) reported that Pyribenzamine has no influence upon the immunization activities of the body or on the chemotherapeutic activity of antibacterial agents.

Boquet (114) showed that the Sanarelli-Schwartzman phenomenon induced by injection of *E. coli* filtrates was not affected by 929 F or Antergan. Antergan also decreases the mortality of guinea pigs exposed to freezing by CO_2 snow (119). Di Macco (120) showed that histamine increased the volume of peritoneal exudate in the guinea pig induced by croton oil and Antergan decreased it.

Reuse (139) reported that Antergan, Neoantergan, 3277 RP, and Benadryl all counteracted histamine-induced spasm of the longitudinal muscles of the stomach of the guinea pig and of the cat.

Antergan, Neoantergan, Benadryl, and 3277 RP decreased the effect of histamine on the isolated perfused cat heart, but the drugs were not as effective in combating this effect of histamine as they were



Graph II.—Dose required to protect most of the animals against lethal histamine aerosol.

N—Neoantergan.
P—Pyribenzamine.
B—Benadryl.
H—Heteramine.

upon isolated smooth muscle. Neoantergan had a potency three times that of Benadryl by this test. On the isolated rabbit heart Neoantergan was more potent than 3277 RP, Antistine, or Benadryl in counteracting the effects of histamine (130).

Capraro (121) reported that Antergan and Benadryl have a dilating action on large arteries, making them insensitive to the action of injected histamine. However, Benadryl has no effect on the

capillaries so that capillary dilatation due to histamine occurs even after Benadryl.

Neoantergan, 25 μg., reduced the dilatating effect of 25 μg. of histamine on the perfused cat heart coronary vessels and on the coronary vessels of the dog heart-lung preparation. Further, the drug abolished the stimulant effect of histamine on the isolated rabbit auricles. Upon the driven rabbit auricles, Neoantergan was twice as active as quinidine in reducing the rate of contraction (97).

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Ungar, Parrot, and Bovet (106) antagonized the spasmogenic effect of histamine with 933 F.

Heteramine, 1 μg./cc. of bathing fluid, prevented the contraction of the isolated guinea-pig ileum usually produced by 1 μg./cc. histamine (46).

Effect on the Uterus.—Selle (123) reported that the effect of 10 μg. of histamine on the isolated guinea pig uterus was prevented by 0.1 mg. of Benadryl.

Sherrod, *et al.* (71), found that Benadryl, Pyribenzamine, and Neoantergan all given intravenously in a 3 mg./Kg. dose counteracted the spasmogenic effect of injected histamine on the dog uterus.

Contraction of the isolated guinea-pig uterus produced by 10–20 μg. of histamine was counteracted by 10–50 μg. of Pyribenzamine (90).

Gayet-Hallion and Quivy (116) reported that

Antergan inhibited the action of histamine on the isolated guinea-pig uterus but this inhibition was abolished by doubling the calcium or magnesium-ion concentration of the bathing fluid.

Dews and Graham (97) found that 0.2 μg. of Neoantergan significantly reduced the response of the isolated guinea-pig uterus to 3 μg. of histamine. They confirmed the results of Bovet and Walthert (92) on the isolated rat uterus, finding that Neoantergan relaxed this organ in the same manner that histamine did. Thienes (111) has observed this same effect with Benadryl and Pyribenzamine.

Reuse (130) has shown that Neoantergan was more potent than either 3277 RP or Benadryl in suppressing the *in vitro* response of the nonpregnant cat uterus to histamine. 3277 RP had to be added to the bath one to two minutes prior to the addition of histamine and even then it was more effective in blocking the second rather than the first dose of the latter. No difference was seen in the response of the pregnant or nonpregnant rabbit uterus to Neoantergan, 3277 RP, Benadryl, or Antistine after histamine. However, the first compound was more potent than the others in its blocking action. Both Neoantergan and 3277 RP had a similar action on the nonpregnant guinea-pig uterus. Halpern (131) had previously observed that 3277 RP had a histamine blocking effect on the isolated guinea-pig uterus.

Lehmann and Young (44) showed that diethylaminoethyl-9,10-dihydroanthracene carboxylate was more potent than diethylaminoethyl-xanthene carboxylate and diethylaminoethyl-fluorene-9-carboxylate in preventing the response of the sensitized guinea-pig uterus to antigen.

Dreyer and Harwood (82) showed that Neoheteramine counteracted the histamine spasm of the isolated cat and guinea-pig uterus and was more effective on the former. Neoheteramine relaxed the isolated rat uterus similarly to Benadryl and Pyribenzamine.

Staub (103) reported that 929 F and 1571 F were effective in preventing histamine spasm of the isolated uterus.

CLINICAL USAGE

Human Pharmacology.—McGavack, Elias, and Boyd (161, 162) reported that Benadryl in the usual doses had no effect upon the body weight or BMR of humans. Upon the central nervous system the drug has a sedative effect. Orally Benadryl had no effect on the eye but local application of a 0.5% solution caused moderate dilatation while a 1% solution produced mydriasis with an interference with accommodation. Doses of 400 mg. returned the respiratory rate and vital capacity of asthmatics to normal. In doses of 300 mg. in normal humans there were no changes in the lungs, pulse rate, heart rate, nail bed capillary loop, electrocardiogram, but in some patients the systolic blood pressure was decreased. Body temperature was unchanged with oral doses of the drug but intravenous administration decreased the body temperature 1° F. The volume of urine and its constituents were not changed nor was there any interference with the concentration or dilution tests. Urea nitrogen and blood NPN were unchanged in the usual doses. There was no change in the hematological constituents or the blood chemistry after Benadryl. Circulation time was unchanged and capillary permeability as measured by the dye method decreased. The gastrointestinal tract was not disturbed in regard to function because the mouth-anus time was unchanged. There was no increase in salivary secretion but the drug did increase gastric acidity in younger patients while it decreased it in the elderly even after histamine stimulation. The oral administration of Benadryl (50–100 mg.) causes a definite decrease in blood pressure. This decrease may be of two or more hours' duration after a single dose (182). Reinstein and McGavack (163) have shown that 400 mg. of Benadryl orally had no effect upon the glucose tolerance curves in young or elderly humans but that 30 mg. of the drug intravenously increased glucose tolerance.

Koepf, *et al.* (95), reported that 3 normal male subjects 22–24 years of age showed no changes in body weight, hematology, blood chemistry, blood pressure, or liver function after receiving 150 mg. of Pyribenzamine orally for eighty days.

Gastric Secretion.—The antihistamine drugs apparently have little or no effect upon histamine-stimulated gastric secretion in man. McGavack, Elias, and Boyd (164) gave 200, 300, and 400 mg. of Benadryl orally daily in divided doses for periods of from two to seven weeks. Upon the basis of predetermined normal gastric acidity levels following the administration of 50 cc. of a 7% alcohol meal, they concluded that the drug had a depressant effect upon gastric acidity. Both free and total acid secretion were depressed but no gastrointestinal symptoms were observed in the 8 normal subjects and the 5 patients with gastrointestinal neurosis.

Moersch, Rivers, and Morlock (165) reported that there was no reduction in the concentration of free hydrochloric acid and no consistently significant reduction in the total volume of secretion following intravenous Benadryl in doses of 60, 100, or 200 mg. Fifteen cases of proved and one case of suspected duodenal ulcer were used as test subjects. The histamine was administered either subcutaneously or intravenously. In 3 patients an arrowroot cookie test meal was used as gastric secretory stimulator rather than histamine. Regardless of the stimulator used the results were the same.

McElin and Horton (166) investigated the histamine-induced rise in gastric acidity in 8 subjects with multiple sclerosis. Three of their patients who received the drug intravenously (40–60 mg.) gave results which suggested that Benadryl inhibited the histamine-induced rise in acidity. Similar results were obtained with one patient who was premedicated orally (400–620 mg.) fourteen hours before the test or intramuscularly (20 mg.). One patient did not respond to histamine and one had a Benadryl-provoked rise in gastric acidity. Two other patients showed no inhibition of gastric acidity by Benadryl regardless of the mode of administration.

Wolf and Wolff (168) reported that Benadryl (50 mg.) increased mucin secretion, viscosity of the secretion, slightly decreased the color, and decreased the total acidity and turgidity of gastric secretion during the phase of average gastric function. However, the drug had no effect during a phase of gastric hyperactivity related to emotional stress. The first effect may have been due to the slowly induced but long acting local vasoconstriction caused by Benadryl.

Serafini and Biozzi (169) found that 2 cc. of a 2% solution of Antergan administered after subcutaneous injection of 1 mg. of histamine HCl in 10 patients had no effect on the hypersecretion of gastric juice.

Decourt (170) found that Neoantergan had no effect on the hydrochloric acid content of gastric juice, or on the gastric response to histamine.

Miscellaneous Effects.—Leavitt and Code (171) tested the anesthetic effect of Benadryl injected intradermally in 10 humans. One forearm was injected with 0.3 cc. of Benadryl (1:500, 1:1000, 1:5000, 1:10,000, and 1:20,000) while the other was injected with procaine (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200). Anesthesia was tested using electric stimulation from an algesimeter. Benadryl was shown to possess definite local anesthetic properties but when injected at concentrations greater than 1:500 it produced tissue necrosis and ulceration in 4 of the 10 subjects tested.

Gelvin and McGavack (38) showed that the blood and spinal fluid concentrations of Benadryl were approximately equal after the administration of oral doses of 100–400 mg. to six human subjects. Gelvin, Elias, and McGavack (172) explain this upon the basis of the unaltered permeability of the meningeal capillaries. The five patients studied received 1900 to 3450 mg. of Benadryl during one week. No changes in the spinal fluid were recognized nor did the disturbances in activity of the central nervous system cause any alteration in the formation or character of the fluid.

Pellerat and Murat (173) showed that subcutaneous injections of either Antergan or Neoantergan caused a 6- to 12-fold increase in the blood histamine levels for more than twenty-four hours. Similar results were obtained by Staub (174) when 0.2 mg. of epinephrine was injected intravenously. However, the intravenous injection of 200 mg. of Antistine prior to the epinephrine prevented or diminished the blood histamine rise.

Acute Urticaria.—Benadryl, in dosage fitted to the needs of the patient, has proved very effective in the treatment of acute urticaria (175, 182–185). In general, the pruritis associated with this condition is relieved within twenty to sixty minutes after the first 50–100-mg. dose of the drug and all lesions disappear within two to six hours (178). Doses of 2–20 mg. two to four times are effective in children (180). Benadryl is effective in the treatment of drug rash (185). Relief of the urticaria and partial relief of the angioneurotic edema caused by insulin sensitivity by the use of 250–350 mg. of Benadryl has been reported by Gastineau and Leavitt (180, 181). Further, Benadryl at various dilutions reduced the flare reaction caused by the intracutaneous injection of 0.2 cc. of insulin.

Benadryl and Pyribenzamine are about equal in the relief of the urticaria, particularly that caused by drug sensitivity (195).

Rauchwerger, *et al.* (198), found that 50 mg. of Benadryl four times daily for three days had no effect on acute urticaria due to streptomycin. However, 50 mg. of Pyribenzamine four times daily gave relief within twelve hours in three patients.

Pyribenzamine, in dosage of 50–500 mg. daily, gives definite relief in acute urticaria, but the drug is only a palliative (188, 188–190, 253).

Goodhill (191) relieved 5 cases of penicillin urticaria with 50 mg. of Pyribenzamine every four to six hours.

Thenylene is beneficial in controlling the pruritis of acute urticaria (194). Comparison of this drug with Benadryl and Pyribenzamine shows it to be equally effective but at a somewhat larger dose (254).

Sixteen of 17 cases of drug and serum reactions were relieved by Thenylene 150–300 mg. daily. These cases included patients sensitive to Thiouracil, Penicillin, streptomycin, and horse serum (195).

Neoantergan is very effective in relieving acute urticaria (176). Hunter controlled the acute urticaria due to penicillin in 5 cases with 300–800 mg. of Neoantergan daily. In 2 cases penicillin was not discontinued while Neoantergan was being given but no rash recurred. Hunter and Hill (193) controlled the urticarial reaction of 8 of 9 patients to liver extract with 200–800 mg. of Neoantergan daily. Reactions to zinc protamine or globin insulin were also controlled by the drug.

Ereaux and Craig (197) reported Anthallan gave prompt relief in drug rashes due to sulfathiazole and penicillin.

Thephorin relieved the itching and urticaria due to both cold sensitivity and food (255).

Antistine produces symptomatic relief for periods of two to twelve hours in acute urticaria. Comparison with Benadryl and Pyribenzamine showed that Antistine was equally effective in treating this condition (256).

Table V gives a comparison of the effectiveness of several antihistamines in acute urticaria.

TABLE V.—EFFECTIVENESS OF ANTIHISTAMINES IN ACUTE URTICARIA

Drug	Daily Dosage, Mg.	Total Number of Cases	Total Number of Cases Relieved
Benadryl	50–350	71	61
Pyribenzamine	50–400	193	157
Thenylene	50–300	12	7
Neoantergan	50–400	13	11
Thephorin	50–350	11	7
Antistine	50–400	10	7

Vasomotor Rhinitis or Hayfever.—This condition, seasonal or nonseasonal, may be controlled with Benadryl but unless the drug is continued the symptoms recur. Relief usually begins within thirty-five to sixty minutes and is of varying duration, often requiring dosage at two-hour intervals (175, 179, 190, 199–204, 210). The drug has been shown to be effective in the treatment of vasomotor rhinitis in children (202). Further, it is more effective in children (2 of 3 cases) than in adults (2 of 11 cases) (179). It has been reported that vasomotor rhinitis is not ameliorated by Benadryl (182).

Pyribenzamine has been used successfully in the treatment of nonseasonal allergic rhinitis (187–189) and in chronic allergic rhinitis (188). Goodhill (191) noted that there was a reduction in the excessive rhinorrhea, a wider airway, and the sneezing, palatal pruritis, and edema ceased or were greatly reduced in patients with acute or chronic allergic rhinitis after Pyribenzamine.

Thenylene gives symptomatic relief in hayfever (195) and some measure of relief in nonseasonal vasomotor rhinitis (194).

Bernstein, Rose, and Feinberg (195) compared Neoantergan, Benadryl, and Pyribenzamine by their effects in patients with hayfever. All the drugs were effective but inasmuch as the total number of cases tested with each drug were not equal it is hard to designate the most effective of the three. Further, some patients responded to one drug but not to the others, showing that the clinician should utilize the drug which gives the desired results regardless of the animal experiments which show that one drug is more potent than another. Further, Criep (206) recently stated that Neoantergan, Thenylene, Benadryl, and Pyribenzamine produced only a palliative relief of vasomotor rhinitis.

Westcott (205) treated perennial vasomotor rhinitis with both Anthallan and Benadryl and found the former compound only 20% effective. Ghiselin (215) reported that Anthallan was effective in relieving seasonal and nonseasonal hayfever.

Antistine also produces symptomatic relief in patients suffering with allergic rhinitis. Further, this drug compares favorably with Pyribenzamine in relieving this condition (256).

Table VI gives a comparison of the effectiveness of several antihistamines in vasomotor rhinitis.

TABLE VI.—EFFECTIVENESS OF ANTIHISTAMINES IN VASOMOTOR RHINITIS

Drug	Daily Dosage, Mg.	Total Number of Cases	Total Number of Cases Relieved
Benadryl	50–300	502	420
Pyribenzamine	50–300	1136	831
Thenylene	100–400	229	141
Neoantergan	50–1500	121	78
Anthallan	85–340	42	42
Antistine	50–400	59	35

Chronic Urticaria.—Benadryl is beneficial in the treatment of this condition but the drug is only a palliative as the itching and wheals recur when the drug is stopped (175, 177, 179, 182, 183, 185, 199, 201, 204, 207). Intravenous injection affords relief when oral medication is ineffective (208). Logan (202) controlled chronic urticaria in children with Benadryl, 2 mg./lb. body weight.

Benadryl was more effective than Pyribenzamine in the treatment of urticaria due to penicillin. Dosage of either drug was 50–100 mg. three times daily (207).

Chronic urticaria responds to Pyribenzamine therapy but continuous medication is required to prevent recurrence of the condition (186–188, 190, 253).

Thenylene has only been tested in a small number of cases but the drug is as effective as other antihistamines in current use (196, 254).

Hunter (192) controlled chronic urticaria associated with angioneurotic edema with Neoantergan. No tolerance developed in three months and the urticaria recurred within ten hours to four days after the drug was stopped.

Schwarzschild (209) reported that Anthallan relieved chronic urticaria and papular urticaria.

Kesten and Sheard (255) found that Thephorin relieved chronic urticaria within a few hours after the first 25–50 mg. dose. Continuous medication did not produce tolerance and the attacks were shorter, less severe, and the itching usually absent.

Friedlaender and Friedlaender (256) showed that although Antistine was only 10% as active as Pyribenzamine by the guinea-pig test, it was fully as effective clinically. However, the effective dose of the latter is twice that of the former.

Table VII gives a comparison of the effectiveness of several antihistamines in chronic urticaria.

TABLE VII.—EFFECTIVENESS OF ANTIHISTAMINES IN CHRONIC URTICARIA

Drug	Daily Dosage, Mg.	Total Number of Cases	Total Number of Cases Relieved
Benadryl	50–500	221	181
Pyribenzamine	50–400	241	138
Thenylene	50–400	18	15
Neoantergan	300–800	8	8
Anthallan	85–340	49	45
Thephorin	100–300	15	15
Antistine	200–400	9	3

Asthma.—Benadryl may or may not be beneficial in bronchial asthma and in general such drugs as ephedrine are more effective (199). Where asthma is associated with hayfever or pollinosis Benadryl has proved to be helpful (175, 179, 182, 201, 202, 204, 210, 240).

Levy and Seabury (212) reported that after the oral administration of 100 mg. of Benadryl there were no consistent changes in vital capacity, tidal air, minute ventilation, expiratory differential, respiratory rate, or degree of emphysema in 16 asthmatics. These tests were performed with a spirometer thirty to sixty minutes after administration of the drug. Curry (213) demonstrated that 10–30 mg. of Benadryl intravenously reduced the bronchio-constrictor effect of 0.02 mg. of histamine intravenously in 2 test subjects. The vital capacity reduction after Benadryl was less than half that produced by histamine alone. Pyribenzamine, 50 mg. orally, had a delayed onset of action in 3 patients, but gave results similar to Benadryl. Systemic effects (flushing, headache, and taste) were partially blocked in all cases.

Epstein (190) reported that asthma was relieved when either Benadryl or Pyribenzamine was given in equal doses. However, in general, results are disappointing. Goodhill (191) had similar results with asthmatics.

Pyribenzamine is no more effective in asthma than Benadryl (187–189, 253). Koepf, *et al.* (214), reported that Pyribenzamine and ephedrine were more effective than either drug alone in the treatment of bronchial asthma.

Feinberg and Bernstein (194) found that Thenylene did not decidedly help the dyspnea of asthma in 30 patients although it did help the preasthmatic-spasmodic cough in 6 of 9 patients. Thenylene is of little benefit in bronchial asthma even in doses of 400 mg. daily (195).

Neoantergan is not very effective in relieving either bronchial asthma or pollen asthma (176). Bernstein, Rose, and Feinberg (195) reported that the incidence of benefit and the degree of relief were only moderate in asthmatics given Neoantergan, Benadryl, or Pyribenzamine. Children generally responded more favorably than adults and the effect was greater on the cough than on the dyspnea.

Antistine benefits the cough to a greater degree than the dyspnea in asthmatics. Where asthma is associated with rhinitis, the latter is benefited even when the asthmatic condition is not. Antistine is as effective as Pyribenzamine in the treatment of asthma, but the results with both drugs are disappointing (256).

Table VIII gives a comparison of the effectiveness of several antihistamines in bronchial asthma.

TABLE VIII.—EFFECTIVENESS OF ANTIHISTAMINES IN BRONCHIAL ASTHMA

Drug	Daily Dosage, Mg.	Total Number of Cases	Total Number of Cases Relieved
Benadryl	50–400	213	101
Pyribenzamine	100–400	327	109
Thenylene	100–400	30	0
Neoantergan	100–400	18	7
Antistine	200–400	24	9

Atopic Dermatitis.—Benadryl benefits pruritis more than atopic dermatitis (179, 182–184, 204).

Epstein (190, 216) found both Benadryl and Pyribenzamine effective in controlling the pruritis associated with atopic dermatitis and drug eruptions in elderly patients. However, the latter was more effective in atopic dermatitis. Bernstein, *et al.* (195), found Benadryl and Pyribenzamine about equal in relieving the itching of atopic dermatitis. The lesions were not consistently benefited by either drug but with a decrease in itching some improvement was noted in the lesions.

Perry (218) reported that topical application of 2% Benadryl ointment relieved only 8 of 22 patients suffering from various pruritis dermatites. However, poor absorption of the ointment was probably the cause of these results.

Pyribenzamine gives relief to the patient suffering from atopic dermatitis (185). Pyribenzamine ointment relieved the itching of atopic dermatitis in 24 of 33 cases (217).

Peirce and Mothersill (196) found that Thenylene was not too effective in counteracting pruritis. However, it has been stated that Thenylene relieves the itching of atopic dermatitis. Thenylene is as effective as either Benadryl or Pyribenzamine in relieving the pruritis associated with atopic dermatitis (254).

In the small number of cases thus far tested, Neoantergan has not been too successful in relieving atopic dermatitis. However, it does relieve pruritis (176).

Schwarzschild (209) found Anthallan gave relief of varying degrees in atopic dermatitis (*neurodermatitis disseminata*). Ereaux and Craig (197) were able to relieve this condition by giving 85 mg. of Anthallan 3 to 4 times daily. Infantile eczema was also benefited.

Thephorin is remarkably effective in allaying itching of atopic dermatitis (255).

Antistine relieves atopic dermatitis and is as effective as Pyribenzamine when tested in the same patient. In some cases one drug was effective after the other had failed to give relief (256).

Table IX gives a comparison of the effectiveness of several antihistamines in atopic dermatitis.

TABLE IX. EFFECTIVENESS OF ANTIHISTAMINES IN ATOPIC DERMATITIS

Drug	Daily Dosage Mg.	Total Number of Cases	Total Number of Cases Relieved
Benadryl	50–150	45	23
Pyribenzamine	50–150	44	30
Thenylene	50–200	19	11
Neoantergan	50–400	14	3
Anthallan	85–340	59	51
Thephorin	150–300	15	7
Antistine	50–400	5	3

Other Conditions.—Goodman and Coonrad (222) have found Benadryl, 100 mg., very effective in the prevention of histamine-induced headache. In 18 patients known to develop headache after histamine injections, Benadryl prevented the condition in fifteen.

Brewster (223) has stated that Benadryl alleviates or abolishes the symptoms of the common cold in both adults and children. A 50-mg. capsule of the drug was given at the first sign of nasopharyngeal soreness and serious discharges were eliminated and the cough reflex inhibited. However, in view of the small number of cases treated, Benadryl should not be considered as a specific in the treatment of the common cold.

Logan (202) relieved a 3-year-old child, suffering from nephrosis, with Benadryl. Schwartz and Levin (175) employed Benadryl, 50 mg. per dose, as a palliative in the treatment of allergies of miscellaneous origin. Hypersensitiveness to cold was reduced by the oral administration of 200 mg. of Benadryl daily for forty-eight days. Regression occurred when the drug was discontinued (219). Benadryl was effective in relieving 2 of 3 cases of cold urticaria and one case of heat urticaria. It also was effective against penicillin reaction in 3 patients (182). Feinberg and Friedlaender (220) tried Benadryl in several cases of rhinitis and asthma which were precipitated by temperature changes. Their results were negative. Benadryl, 200–300 mg. daily, gave relief to 4 patients with scleroderma and to 9 patients with acrosclerosis (183, 184). Lynch (185) found Benadryl effective in the treatment of pruritis vulvae, insect bites, and erythema multiforme. Lofstrom and Nurnberger (224) found that 50–150 mg. of Benadryl intravenously or 50–200 mg. orally counteracted the symptoms of radiation sickness. Epstein (190) successfully treated pruritis ani and vulvae and drug rash with Benadryl and Pyribenzamine 50 mg. three or four times daily.

Baer and Sulzberger (186) relieved 13 of 28 cases of pruritis associated with miscellaneous dermatoses by the oral administration of 50–300 mg. of Pyribenzamine daily. Pyribenzamine, 50–100 mg. per dose, prevented hypersensitiveness to cold (187). Feinberg and Friedlaender (188) found Pyribenzamine of benefit in unclassified dermatitis, drug rash, penicillin allergy, chronic headache, gastrointestinal allergy, and pruritis vulvae. However, even at a dose of 100 mg. four times daily the drug was only a palliative. Penicillin urticaria in a syphilitic was controlled by Pyribenzamine 40 mg. every two hours (221). Topical application of 2% Pyribenzamine ointment relieved the itching in 3 cases of dermatitis of unknown origin and 8 cases of pruritis ani (217). Fischel (132) reported that 25–50 mg. of Pyribenza-

mine daily had no effect on the Arthus reaction induced by purified bacterial antigens injected into adults and children.

Peirce and Mothersill (196) reported that 200 mg. of Thenylene daily relieved sensitivity to cold, had no effect in hypertensive headache or histamine-induced headache, but gave moderate relief in functional headache. Thenylene was as effective as either Benadryl or Pyribenzamine in the treatment of anal and vulval pruritis (254).

Neoantergan, 50–400 mg. three times daily, has not been found to be beneficial in the treatment of angioneurotic edema, fungus infection of the skin, gold dermatitis, poison ivy dermatitis, or vernal conjunctivitis. However, when more cases have been treated this may prove to be incorrect (176).

Thephorin, 150–300 mg. daily, relieves erythema multiforme, papular urticaria, neurodermite, and pruritis ani, but not psoriasis. Hypersensitiveness to cold was also relieved (255).

Antistine, 200–400 mg. daily, was not found to be beneficial in pruritis ani or dermatitis of unknown origin. However, more cases must be investigated before any conclusions can be drawn (256).

Contact Dermatitis.—Levin (179) found Benadryl partially effective against contact dermatitis and drug rash. Friedlaender and Feinberg (182) found that only the pruritis associated with this condition was controlled with Benadryl.

Feinberg and Bernstein (217) relieved two cases of contact dermatitis with Benadryl and Pyribenzamine.

Peirce and Mothersill (196) successfully relieved 3 of 4 cases of contact dermatitis with 200 mg. of Thenylene daily. Neurodermatitis was also slightly benefited.

Histamine Flare Reaction.—Friedlaender and Feinberg (182) reported only a slight inhibition to intradermal histamine (1:1000–1:100,000) after oral administration of 50 mg. of Benadryl or Pyribenzamine. A similar inhibition is also produced against ragweed, mechanical, or chemical whealing. A marked reduction was obtained when the antihistamine was applied locally to the injection site. Elias and McGavack (225) found that 300–400 mg. of Benadryl daily reduced the erythema and wheal reaction caused by the direct application of one drop of 1% histamine to the volar surface of the forearm. Premedication with Benadryl enabled Feinberg and Friedlaender (220) to perform scratch tests for sensitivity to various substances because the drug suppressed the dermographic response previously obscuring the results. The dosage was regulated to fit the needs of the 20 adults and 10 children tested.

Grob, Lilienthal, and Harvey (226) found that the histamine flare and wheal reaction produced by the intracutaneous injection of 0.05 cc. of curare or *d*-tubocurarine was inhibited by prior injection of 150 μg. of Pyribenzamine or 1.5 mg. of Benadryl. Mixing of the drugs before injection gave the same results, but the effect was less when 0.1 mg. of histamine was used instead of curare or *d*-tubocurarine. Intra-arterial injection of 10 mg. of Pyribenzamine reduced the hyperemia, venous engorgement, and ecchymotic discoloration produced by the intra-arterial injection of 50 units of curare. The usual temperature rise associated with curare injections was also inhibited. Oral administration of 200 mg. of Pyribenzamine at one hundred and thirty minutes prior to the injection of 50 units of curare had the same effect.

Arbesman, Koepf, and Miller (227) observed a definite reduction in the histamine wheal and flare produced by both serial dilutions of histamine and allergens after 50–150 mg. of Pyribenzamine orally. A reduction in the reactivity of skin sites passively sensitized with serum containing cottonseed reagins was also observed.

Baer and Sulzberger (228) found that 150 mg. of Pyribenzamine orally reduced the dermographic response of a sensitive patient to a standard stimulus. Claman (229) showed that 150 mg. of Pyribenzamine definitely reduced the histamine flare reaction due to ragweed or histamine.

Feinberg and Freidlaender (188) noted definite improvement in 14 of 16 patients with dermographism after the administration of 50–100 mg. of Pyribenzamine.

Kurtin, Bierman, and Yontef (231) have shown that the iontophoresis of 5% Pyribenzamine prevents the erythema, edema, and vesiculation of normal patients exposed to ultraviolet light. They suggest that the drug be used in treatment of sunburn.

Feinberg and Bernstein (194) found Thenylene of benefit in 7 of 9 cases of dermographism, one case of pruritis of unknown origin, and 2 of 3 cases of pruritis ani. The experimental wheal and flare reaction of histamine or a specific antigen was decreased by local application of the drug.

Parrot (232, 233, 234) has shown that Antergan, 200–400 mg. per dose, inhibits the skin reaction due to burning, friction, viper venom, electrical stimulation, or injected histamine. Warembourge, Linquette, and Machon (235) have confirmed these results.

Thephorin, 50 mg. subcutaneously, prevented the itching but not the wheal formation caused by local application of one drop of 1:1000 histamine. Similar results were obtained with antigens after oral administration of the drug. In testing patients giving a positive dermographic reaction the same results were obtained (255).

Dysmenorrhea.—Eyermann (199) reported that dysmenorrhea was not ameliorated by Benadryl 50 mg. three times daily.

Serum Sickness.—Logan (202) found that 30 mg. but not 20 mg. of Benadryl were effective in treatment of a serum reaction in a 3-year-old child. Peterson and Bishop (230) reported that Benadryl, 50–100 mg. every six to eight hours, was effective in the treatment of serum sickness due to impure and pure horse serum and rabbit serum preparations.

Shock reactions due to injected allergens or liver extract were prevented by 100 mg. of Pyribenzamine prior to the injection. However, the drug was only a palliative (187).

Thephorin gave rapid relief to two patients suffering from serum sickness due to tetanus antitoxin injections (255).

Antistine has been used unsuccessfully in treating one case of "serum sickness" type reaction due to penicillin (256).

Migraine.—Benadryl, 50 mg. three times daily, had no effect on migraine (199). Barnett, *et al.* (201), obtained uncertain effects with Benadryl, 50–150 mg. daily, in two patients with migraine. Williams (200) found Benadryl of little value in myalgia

of the head. Migraine in adults was relieved with Benadryl 50–100 mg. every four to six hours. Similar results were obtained in children with a dose of 5–20 mg. two to four times daily. Although Levin (179) was successful in 9 of 15 adult cases and 2 of 3 children, he found that Benadryl aggravated the condition in two instances. Waldbott (204) used Benadryl to relieve 2 of 4 cases of migraine.

Arbesman, *et al.* (187), found Pyribenzamine of little value in migraine or histamine cephalalgia. This has been confirmed (253).

Neoantergan is of little value in the treatment of migraine (176).

Allergic headache is generally not benefited by Antistine (256).

Meniere's Disease.—Williams (200) obtained no results in the treatment of two cases of Meniere's disease with Benadryl.

Pyribenzamine was not beneficial in Meniere's disease (187). Epstein (190) also found that Pyribenzamine and Benadryl were ineffective in treating Meniere's disease.

Allergic Conjunctivitis.—Barnett, *et al.* (201), obtained improvement in 7 cases with Benadryl, 50–150 mg. daily. Benadryl gave complete relief to 2 patients and partial relief to one suffering from this condition (204).

Friedlaender and Friedlaender (256) showed that local application of a 0.25–0.5% solution of Antistine gave symptomatic relief in allergic conjunctivitis. The work confirmed the earlier observations of Bourquin (257).

Toxicity.—In general, toxicity due to overdosage or idiosyncrasy to antihistamine drugs is the same The symptoms commonly observed are dizziness, jitteriness, somnolence, dry nose, nausea, weakness, palpitation, insomnia, abdominal cramping, numbness, cold extremities, fatigue, tendency to hemorrhage, chloroform taste, faintness, acute hysterical reaction, dilated pupil, stupor, narcolepsy, sore tongue, hot flushes, early menses, dermatitis medicamentosa (175–177, 183, 184, 187, 192, 194–196, 206, 236–240, 253–256).

Brown (258) has shown, in a controlled study, that there is a definite psychic factor to be considered when evaluating the side effects caused by antihistamines. He found that placebos caused almost as many responses as Pyribenzamine.

Geiger, Rosenfield, and Hartman (241) reported upon the use of Benadryl in the treatment of pruritis associated with a generalized seborrheic dermatitis of unknown origin. The patient complained of palpitation, dimmed vision, malaise without drowsiness, and heartburn with nausea after receiving a total of 300 mg. of the drug in three days. After the administration of an additional 50 mg. of the drug the patient was found unconscious in bed, cold, pale, pulseless, and blood pressure could not be obtained, eyes were staring but pupils were equal and reacted to light. The administration of 0.46 cc. of a 1:1000 epinephrine solution subcutaneously caused the pulse to become palpable within thirty minutes and the patient became normal in three hours. Four days later the patient again was given Benadryl (50 mg. three times daily) and when 300 mg. had been administered the milder symptoms recurred.

Weil (242) observed acute toxicity due to Benadryl in a 3-year-old boy who had received 150 mg. of the drug in a period of six hours. Twenty minutes after receiving the last 100 mg. the child was found stitting up in bed, singing, laughing, starry-eyed, and in an apparent drunken condition. This was followed by muscular twitchings of the face, involuntary spastic movements of the extremities, and urinary incontinence. The muscular movements became worse, the child became irrational and dove out of bed on his head, his speech became slurred and hypertonic patellar and triceps reflexes were abnormal (all others were normal). The administration of 90 mg. of Seconal orally produced sleep but the muscular hyperactivity was such that the child had to be restrained. He became normal the following afternoon, sixteen hours after taking the last dose of Benadryl.

Schwartzberg and Willerson (243) observed toxicity in a man 38 years old who was taking 50–150 mg. of Benadryl daily. The usual toxic symptoms in varying degrees occurred along with an increased amount of flatus and three stools daily. Physical examination revealed a blood pressure of 100/64, edema of the eyelids, tenderness over the median nerve in both forearms and questionable edema of the forearms and hands. Reflexes were normal and except for the above the patient appeared normal. Even after the drug had been discontinued it was three months before the patient was completely recovered from the effects of this self-medication.

Borman (244) reported on an 18-year-old woman who took about 2000 mg. of Benadryl in forty-eight hours. Lethargy, mental confusion, and disorientation were observed but other than those symptoms of toxicity the woman was normal. Complete recovery occurred in forty-eight hours after stopping the drug and giving fluids freely, combined with several cups of strong coffee.

Swartz (245) treated a patient 32 years old for hayfever with Benadryl and observed the usual side reactions due to the drug. However, after a total dosage of 550 mg. of the drug the following symptoms occurred: nausea, vomiting, sense of rotation upon opening his eyes, marked rapid nystagmus, bilateral tinnitus, cold clammy skin wet with perspiration, blood pressure 90/60, and vertigo. Benadryl was stopped and $^3/_8$ gr. of ephedrine administered orally every two hours. Patient had same symptoms the following day when 9 Gm. of ammonium chloride daily was substituted for the ephedrine. He was placed on a high-protein, high-potassium, salt-free, and fluid-restricted diet. Upon the third day the symptoms were slightly abated but the blood pressure remained 90/60. The patient was not entirely free of the labyrinthine symptoms until the tenth day after stopping the Benadryl. Seven days after recovery the patient was given 20 mg. of Benadryl on an empty stomach and within thirty minutes the symptoms of toxicity returned. Treatment consisted of 0.3 cc. of epinephrine every half-hour for two hours. He was not symptom-free until four hours later. Four weeks later the patient took 25 mg. of Benadryl with the same results within thirty to forty minutes. This time the symptoms remained for eight hours but were controlled with epinephrine.

A case of a 3-year-old girl who swallowed 700–800 mg. (50–60/Kg.) of Benadryl was reported by Duerfeldt (246). The usual symptoms of acute toxicity

were observed and in addition there were convulsions and respiratory paralysis (4 times in less than four hours). Artificial respiration maintained the respiration and rectal administration of ether controlled the convulsions. The ataxia required four days to disappear but repeated examinations revealed no brain damage.

Sternberg (247) reported that a student nurse being treated for hayfever with Benadryl (50 mg. four times daily) experienced depression and confusion. On her day off she took three extra doses becoming drowsy, weak and amnesic, then hysterical a few hours later. For three days she had the same symptoms, crying spells, rubbed her eyes violently, and slept in spells. All this time the original dosage of the drug was continued. When the drug was stopped recovery occurred within forty-eight hours.

Eczematoid dermatitis from the ingestion of Pyribenzamine has been reported by Harris and Shure (248). After taking 50 mg. three times daily for six days the woman experienced a vesicular dermatitis on the dorsal surfaces of both hands, anterior surfaces of both thighs, and on the anterior surface of the chest. Also there was an intense pruritis. This dermatitis could be produced at will with the administration of 50 mg. of the drug, but skin tests were all negative. Epstein (249) observed a similar reaction in two patients who were receiving 50 mg. of Pyribenzamine three times daily. One developed a dermatitis resembling pityriasis rosea and the other a widespread erythematopapular eruption.

The development of fever and granulocytopenia in an elderly patient who was taking 200 mg. of Pyribenzamine daily was reported by Blanton and Owens (250). Scratch tests were negative but *in vitro* tests indicated that the drug was causing destruction of the leucocytes. The total leucocyte count and differential percentage returned to normal twelve days after the drug was stopped.

Kern (259) reported a case of toxicity due to Pyribenzamine in which the patient fainted after taking one dose of the drug. She continued the drug for a total of three doses, having the same difficulty after each dose. Although she remained prostrate all that day, recovery occurred the morning following withdrawal of the drug.

Peirce and Mothersill (196) gave 5 patients an average daily dose of 170 mg. of Thenylene for a period of three months. They found no evidence of damage to the blood, heart, liver, or kidneys.

Ghiselin (215) reported that Anthallan in total average dose of 11.65 Gm. over a period of seven of thirty-five days had no effect on either the blood chemistry or hematological constituents of the patients treated.

SUMMARY

A review of chemistry, pharmacology, and clinical usage of the antihistaminic drugs has been presented. It is difficult to compare the antihistaminic properties of the drugs because the methods employed by the various investigators were not always the same. From a general pharmacological viewpoint more investigation should be undertaken to determine the site of action of the drugs alone upon the various body systems. Clinically the antihistaminic drugs appear to be beneficial in the treatment of hayfever, vasomotor rhinitis, drug rashes, pruritis, contact dermatitis, acute and chronic urticarias, atopic dermatitis, and allergic conditions causing a histamine flare reaction. However, the results obtained in the treatment of asthma and in histamine-induced gastric secretion are disappointing and indicate that the drugs are either not true antihistaminics, are unable to reach the site of action of histamine on the particular body systems involved, or that some other agent causes the symptoms attributed to histamine. The toxic effects caused by all these drugs are similar and the physician as well as the patient should bear in mind that excessive dosage is dangerous.

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The Quantitative Determination of Crystalline Penicillin G by Infrared Analysis*

By EDWARD A. GARLOCK, JR., and DONALD C. GROVE†

The quantitative analysis of commercial samples of crystalline sodium, potassium, and buffered penicillins for the penicillin G content by means of the infrared spectrometer is presented. The analysis is conducted on a suspension (mull) of the crystals in liquid petrolatum. The proportion of the liquid petrolatum and the crystalline penicillin is kept constant and a cell of known thickness is used, thus eliminating the necessity of an internal standard. The same mull can also be used to identify other types of penicillin present in the sample. The spectra for potassium penicillin G, dihydro F, F, K, and X are given. Results obtained using the infrared technique and the N-ethyl piperidine method for the determination of penicillin G are compared.

THE COMMERCIAL production of infrared spectrometers has made easily available to the chemist a new tool with which not only the qualitative characterization of organic molecules but also quantitative determination of them in mixtures can be obtained. The application of infrared analysis to the crystalline penicillins, both from a qualitative as well as quantitative aspect, has been reported by Barnes, *et al.* (1). In their procedure, the sample of penicillin is analyzed in the solid phase after mulling with liquid petrolatum between rock-salt plates. Quantitative analysis of sodium penicillin G is made, using *dl*-alanine as an internal standard. The purpose of the internal standard is to eliminate the necessity of having to know the length of the absorption cell. The analytical results for known mixtures of sodium penicillin G and magnesium oxide are reported, but no results are given for the determination of sodium penicillin G in the presence of known amounts of the other penicillins. Errors due to crystal orientation and difficulties of thoroughly mixing the sample and standard are discussed.

EXPERIMENTAL

It has been our experience in following the technique of Barnes, *et al.* (1) that variations in the thickness of the mull between the rock-salt plates are not fully compensated for by the internal standard. If the film is a little too thick or too thin, inconsistent results are obtained. In addition, it is difficult to obtain a mull of a fixed ratio between the two solid phases and the liquid petrolatum. Furthermore, examination of many commercial samples of crystalline sodium penicillin, crystalline sodium penicillin buffered with sodium citrate and crystalline potassium penicillin has shown variable absorption in the wave-length region chosen by these authors to measure the absorption of *dl*-alanine. One of the purposes of the present paper is to report a technique in which an absorption cell of known length is employed obviating the necessity of an internal standard.

Analysis of crystalline compounds in the solid state in the infrared is complicated by physical factors not encountered in the case of liquids, solutions, or gases. Reflection and strong scattering of the incident light, the type and size of the crystals as well as the nature of amorphous or crystalline impurities may play important roles. It thus becomes necessary to follow an exacting and standardized technique in order to obtain accurate and reproducible results.

Absorption Cell.—The absorption cell used consists of two circular rock-salt plates two inches in diameter, separated from each other by means of a brass spacer. The cell is held tightly between two metal plates (cell holder) by means of threaded studs and nuts. The metal cell holder is illustrated with dimensions in Fig. 1. A brass spacer was

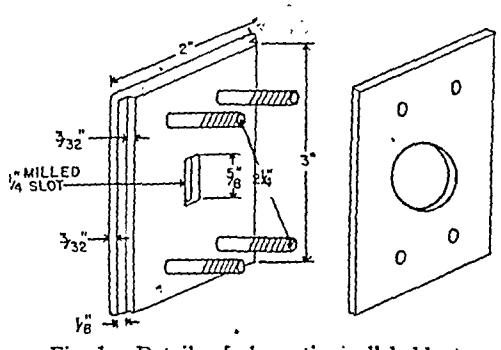

Fig. 1.—Details of absorption cell holder.

chosen in preference to the usual lead spacer because it is stiffer, thus more easily handled; in addition, it can be used repeatedly without change in thickness. The spacer thickness should be between two- and three-thousandths of an inch. The exact thickness need not be known if the same spacer is used for both the standard and the unknown sample. The spacer is cut in the shape of a circular gasket with a 1-inch center hole and a slit to permit the escape of air when the two cells are clamped together. In

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† We wish to express our appreciation to Chas. Pfizer & Co., Inc., for samples of sodium penicillin K and dihydro F; to American Cyanamid Co. for samples of sodium penicillin F and X; and to Commercial Solvents Corp. for the sample of potassium penicillin G.

order to further standardize the procedure, the ratio of the penicillin to liquid petrolatum is kept constant. A satisfactory mull is obtained by using a ratio of one part of penicillin to two to three parts of liquid petrolatum. Since the concentration and cell thickness are fixed, it is possible to measure the penicillin G content of crystalline penicillin from the infrared absorption at only one wave length. If desired, the complete infrared spectrum can be run and the amounts of other types of penicillin present can be estimated at the wave length characteristic for the type.

Procedure.—The data presented here were obtained, using a Perkin Elmer model 12-B infrared spectrometer. The details of the procedure used are as follows:

1. Grind the sample to be analyzed with a mortar and pestle.
2. Weigh by difference 100–150 mg. of liquid petrolatum into an agate mortar. Multiply the actual weight of the liquid petrolatum by 0.4 and add exactly this amount of penicillin to the liquid petrolatum in the mortar.
3. Mix the sample and liquid petrolatum with a small spatula and then mull thoroughly with the pestle until a uniform consistency is obtained.
4. Lay the cell holder down with the studs up and place a 2-inch diameter salt plate between the studs. Center this plate with respect to the opening cut in the holder.
5. Lay the spacer (0.0024-inch thickness) on the plate (see Fig. 2).

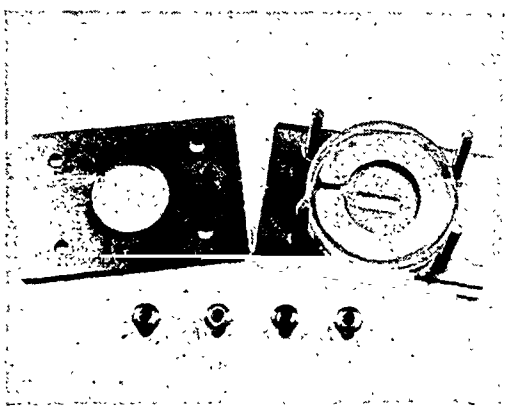

Fig. 2.—Photograph of absorption cell without the mull.

6. Put a small drop of the mull in the center of the plate, and bring the top salt plate down gently so as to spread the mull uniformly over the slit opening (see Fig. 3).
7. Place the metal cover over the assembled cell and screw the cover down tightly. (The spacer prevents change in thickness.)
8. Examine the mull by holding the assembled cell up to the light. It should appear smooth and free of any air bubbles and not in contact with the spacer. If it is not uniform or is in contact with the spacer, dismantle the cell, remove the mull, add a new drop and reassemble as indicated above.
9. Adjust the amplification of the spectrometer to full-scale deflection for one microvolt. Set the slit opening to 0.750 mm. and record the spectrum from 14.8 to 13.8 microns, being sure to take a zero reading (shutter closed) at the beginning and at the end of the run.

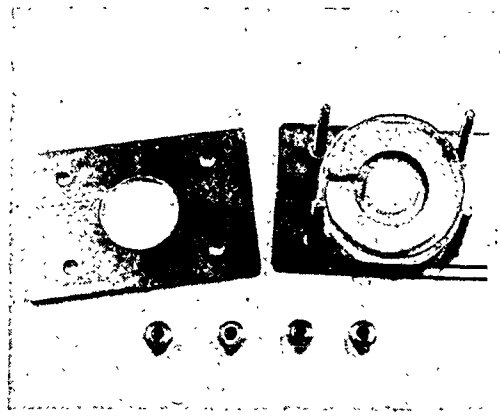

Fig. 3.—Photograph of absorption cell with th mull.

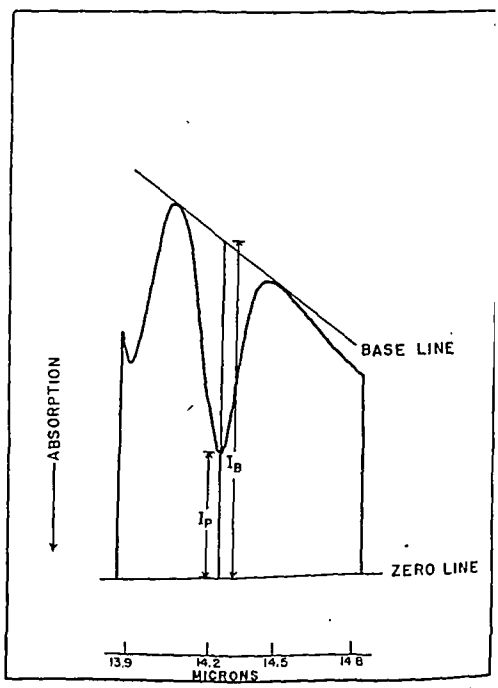

Fig. 4.—Method of calculating the base-line density for crystalline sodium penicillin G.

10. Draw in the base-line between two points, one on each side of the analytical band. As illustrated in Fig. 4, calculate the base-line optical density, Wright (2), Heigl, *et al.* (3), from the equation:

$$D_B = \log_{10} \frac{I_B}{I_P}$$

where

D_B = base-line optical density.
I_P = distance from the zero-line to the maximum absorption of the band.
I_B = distance from the zero-line to the base-line measured at the same wave length as I_P.

Various combinations of the F.D.A. working standard of crystalline sodium penicillin G with either sodium penicillin F, sodium penicillin K, or sodium penicillin dihydro F were prepared. The base-line density of these mixtures was determined by the above method and when plotted against the concentration of sodium penicillin G, a linear relationship was obtained. The curve obtained by mixing the F. D. A. standard sodium penicillin G with a composite mixture of sodium penicillin F, K, and dihydro F is shown in Fig. 5. The appearance of the absorption band at the various concentrations of penicillin G is shown in Fig. 6. Thus, the determination of penicillin G is valid in the presence of penicillins F, K, and dihydro F. Sodium penicillin X which absorbs strongly in the region of 14.2 microns would cause some interference; however, as crystalline penicillin now being commercially produced does not appear to contain penicillin X, this presents no immediate problem. Penicillin X also shows absorption at 12.03 microns (Barnes, *et al.* (1)), and by measuring at this wave length a correction for the amount of penicillin X present could be made. The accuracy of the above method as determined by the analysis of known mixtures of sodium penicillin G and non-G is within 2.5%.

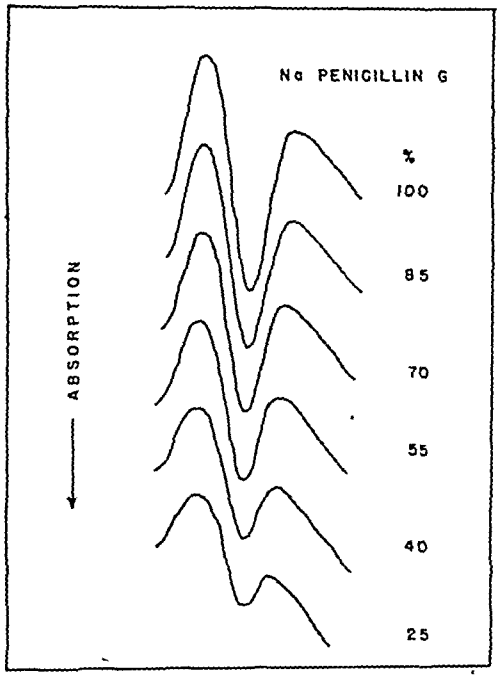

Fig. 6. Infrared absorption at 14.2 microns of crystalline penicillin G when mixed with non-G penicillin.

Base-line density values obtained for eight analyses of the standard sodium penicillin G during a period of two months, using the same cell and spacer, are as follows: 0.414, 0.398, 0.393, 0.402, 0.400, 0.404, 0.398, and 0.395. Thus for accurate routine work it is necessary to determine the base-line density of a standard sodium penicillin G from time to time to take care of changes in the spectrometer and the salt plates of the cell.

Table I shows the precision of this procedure. These results were obtained by analyzing each of two commercial samples of sodium penicillin G four times.

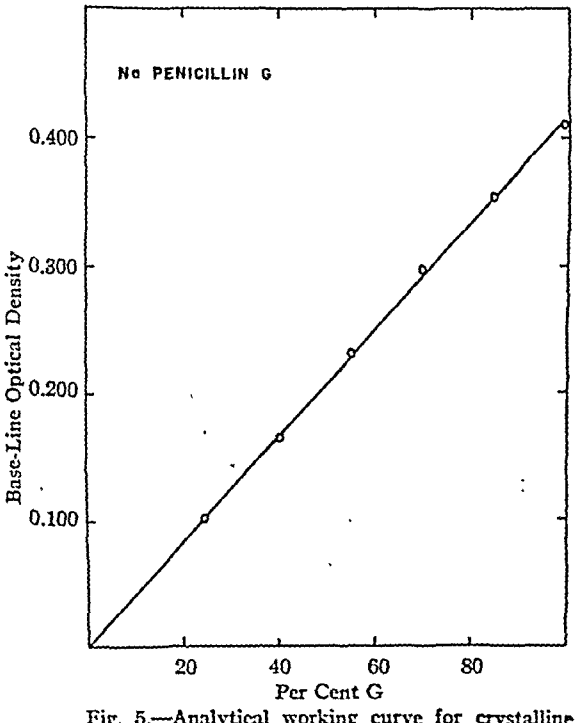

Fig. 5.—Analytical working curve for crystalline sodium penicillin G.

TABLE I—INFRARED ANALYSIS OF TWO COMMERCIAL SAMPLES OF PENICILLIN G

	Sample A, % G		Sample B, % G
	101.1		91.5
	97.2		95.0
	97.0		94.7
	101.7		93.5
Av.	99.3	Av.	93.6

The deviation of these values from the average is within 2.5% for both samples.

A comparison of values obtained for various commercial samples of crystalline sodium penicillin G by the above procedure compared with those obtained

using the N-ethyl piperidine (N. E. P.) method (4) is shown in Table II. Two different lots were run from each manufacturer.

In order to extend this method of analysis to include potassium penicillin G, it was necessary to obtain the various potassium penicillin salts of the other types of penicillin. We have converted the sodium salts of penicillins K, F, dihydro F, and X to the corresponding potassium salts by way of the free acids followed by the formation and crystallization of the potassium salts. All of these salts were crystalline and possessed the calculated biological activity, indicating a high degree of purity. However, because of the scarcity of the sodium salts of these penicillins, we were not able to characterize these potassium salts by more complete analytical data or establish their chemical purity. The units per mg., as determined by bioassay, were 2020 for the potassium penicillin K, 1684 for the dihydro F, 743 for the X, and 1341 for the F.

TABLE II.—COMPARISON OF THE N-ETHYL PIPERIDINE AND INFRARED PROCEDURES USING SODIUM PENICILLINS

Manufacturer	% G, N. E. P.	% G, Infrared	Difference, I. R. − N. E. P.
A	97.0	100.0	+3.0
	95.0	93.8	−1.2
B	95.7	92.5	−3.2
	99.0	101.0	+2.0
C	90.8	85.0	−5.8
	90.8	83.7	−7.1
D	92.0	83.0	−9.0
	81.0	79.6	−1.4
E	91.0	89.5	−1.5
	96.8	99.3	+2.5
F	92.6	96.5	+3.9
	91.5	93.6	+2.1
G	99.6	97.5	−2.1
	97.8	98.5	+0.7
H	88.0	85.5	−2.5
	92.5	92.0	−0.5
I	95.0	96.0	+1.0
	90.0	85.5	−4.5
J	96.0	103.0	+7.0

The potassium penicillin G used in this work was supplied by one of the manufacturers who reported the following analytical data:

Potency

Biological Assay.....1587 u./mg.
Iodometric Assay.....1591 u./mg.

"G" Analysis

Craig extraction.....100.0%
N. E. P. weight.....100.4%
Ultraviolet.....100.0%

The infrared spectra of these potassium penicillins are shown in Figs. 7 and 7*a*. These are the actual recordings of the spectra, using a motor-driven slit control mechanism which gives a nearly level energy

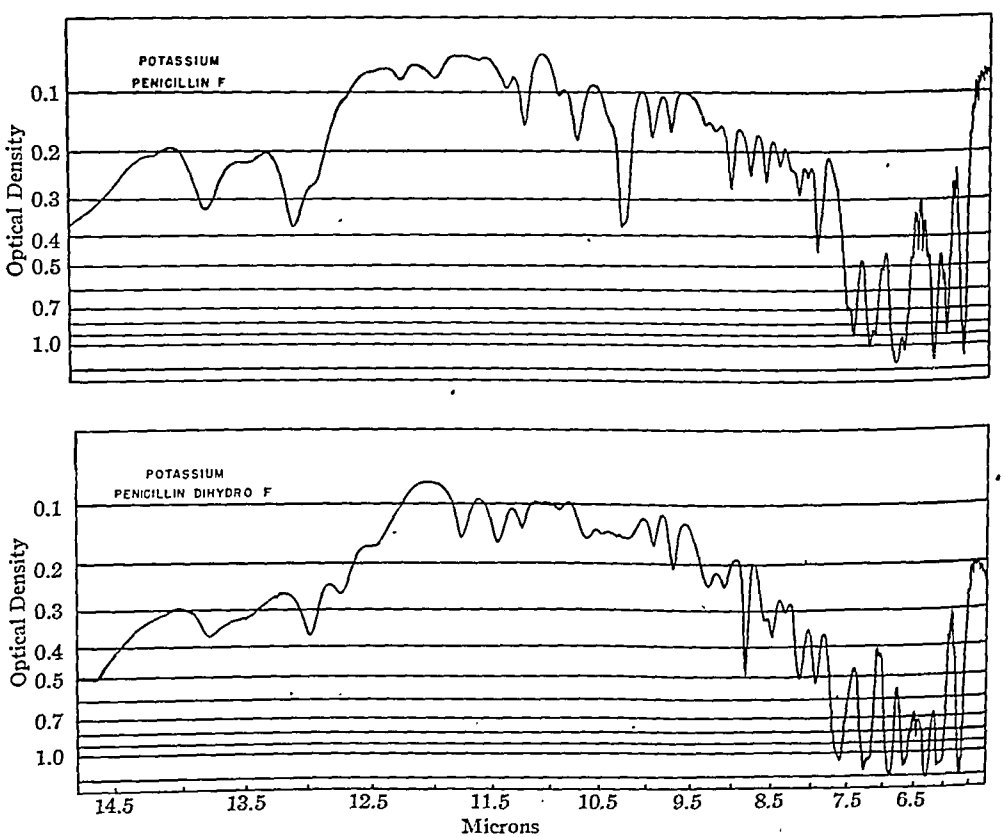

Fig. 7.—Infrared spectra of crystalline potassium penicillins.

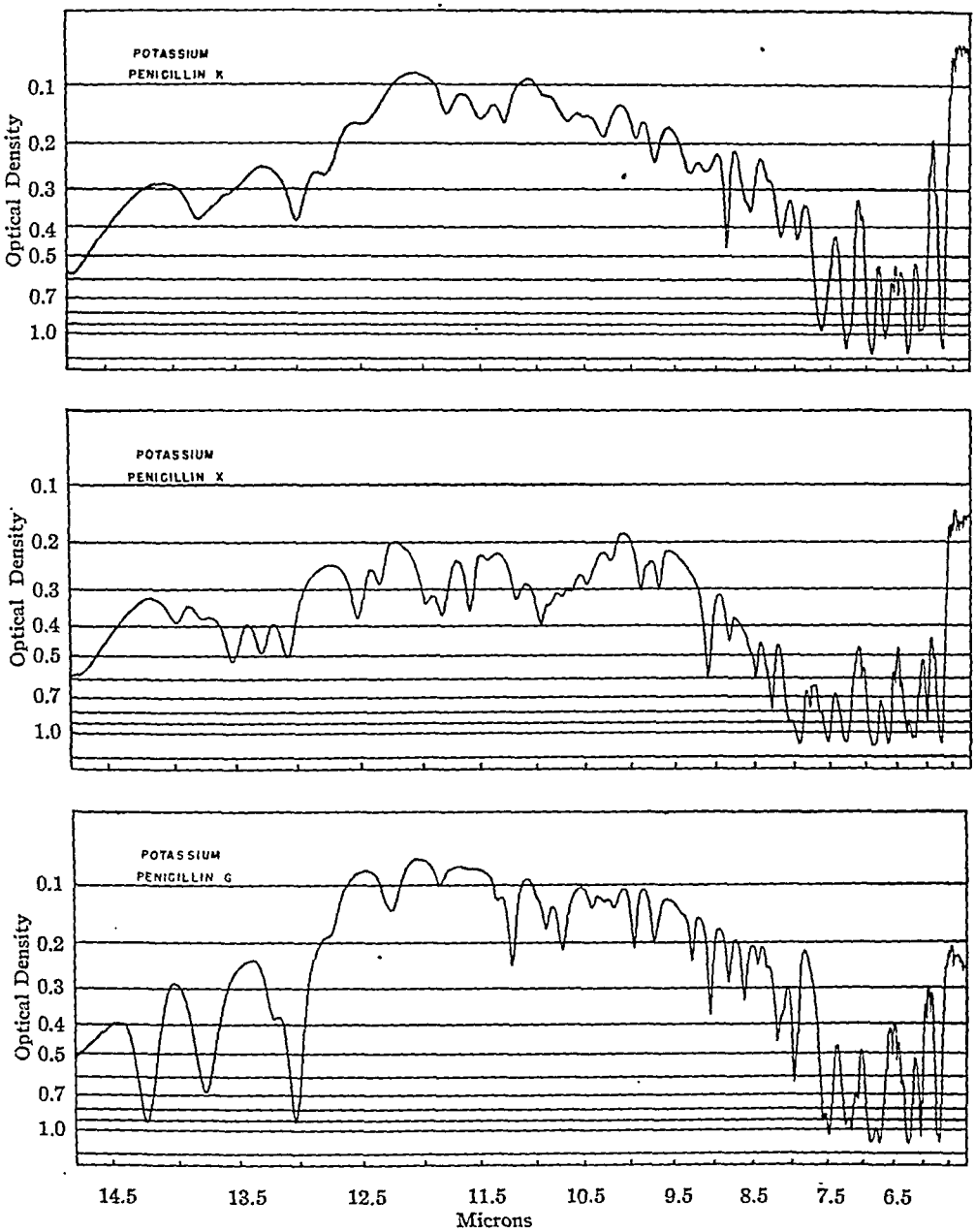

Fig. 7a.—Infrared spectra of crystalline potassium penicillins.

background. The approximate optical densities are indicated on the recordings.

Using the procedure described above for sodium penicillin G, a working curve was prepared for potassium penicillin G diluted with potassium dihydro F. The results show a linear relation between the base-line optical density and percentage of penicillin G. A comparison of the values obtained by analyzing a series of commercial samples of crystalline potassium penicillin G by the infrared procedure and by the N-ethyl piperidine method is given in Table III. The results are slightly higher by the infrared method.

Commercial samples of crystalline sodium penicillin G buffered with sodium citrate prepared by three manufacturers were analyzed for penicillin G content by this infrared procedure. Good results were obtained on the samples of two of the manufacturers while the samples of the third manufacturer gave inconsistent results. These latter samples appeared to have been ground to a very fine powder which would not give a smooth liquid petrolatum mull and consequently gave poor analytical results. However, after recrystallization from 95% methyl ethyl ketone, satisfactory analyses were easily obtained.

TABLE III.—COMPARISON OF THE N-ETHYL PIPERIDINE AND INFRARED PROCEDURES USING POTASSIUM PENICILLINS

Manufacturer	% G, N. E. P.	% G, Infrared	Difference, I. R. − N. E. P.
A	97.0	99.0	+2.0
	97.0	99.0	+2.0
B	96.0	97.0	+1.0
	98.0	98.5	+0.5
C	96.0	94.5	−1.5
	95.5	97.5	+2.0
D	86.0	92.5	+6.5
	90.0	91.0	+1.0
E	94.5	97.0	+2.5
	99.0	98.0	−1.0
F	89.5	96.0	+6.5
	91.5	96.5	+5.0

DISCUSSION

Advantages.—The proposed infrared method has the following advantages:

1. The sample is analyzed in the crystalline state, thus it is not subject to any possible changes that might take place in extraction procedures.
2. An accuracy of about ±2.5% can be obtained for all percentages of penicillin G when mixed with known non-G penicillins other than penicillin X.
3. Both a qualitative and quantitative analysis can be made on the same mull, thus a knowledge of the other types of penicillin present can be obtained.
4. An estimation of the amount of non-G penicillins can be made if they are present to the extent of 5–10% or more.
5. The method is fast and the technique is not difficult.

Disadvantages.—It may be said that some of the possible disadvantages are:

1. The necessity of having pure standards for each type penicillin salt and having to redetermine the base-line densities for the standards at various intervals.
2. Difficulty may be experienced in preparing a satisfactory mull, if the sample has been milled to extremely small particle size. In this case, one has to resort to recrystallization.
3. Uncertainty as to the effects of degradation products and accompanying impurities in commercial samples. This might include compounds such as salts of penicilloic acid, penillic acid, etc., which were not available for this study.

This method gives results which are in agreement with the N-ethyl piperidine procedure when samples of high G content are compared. The N-ethyl piperidine method requires careful attention to details (i.e., careful selection of solvents and reagents, control of the temperature of solutions and the time and temperature of precipitation) and, like other gravimetric procedures, it is subject to solubilizing and coprecipitation effects. It has been observed in this laboratory that the presence of penicillin K in amounts of 10% or more produces a solubilizing effect on the precipitate in the N-ethyl piperidine method resulting in low values for penicillin G. We have also observed that the presence of 10% or more of penicillin F or dihydro F may cause coprecipitation, thus giving rise to high penicillin G values. The amount of the coprecipitation varies with temperature, different batches of solvent used, and different lengths of time the precipitate is allowed to stand.

SUMMARY

The quantitative infrared analysis of penicillin G in commercial samples of crystalline sodium, potassium, and buffered penicillins has been described. A mull of definite concentration is analyzed in a cell of constant thickness, thus eliminating the necessity of an internal standard. The prepared mull is suitable for both qualitative and quantitative analysis. The absorption spectra of the potassium salts of the penicillins are recorded. A comparison of this infrared method with the N-ethyl piperidine method has been made.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Epicarin
Mercaptopyruvic acid
Quinitol
Ribose-*l*-phosphate
Dambonitol
Sequoytol
Scyllitol
Conduritol
Inosinic acid
Xanthylic acid
Uridine
Cytosine desoxyriboside

Aseptic Operation and Control of Ampul Filling Rooms*

By JOHN H. BREWER

In our attempts to maintain sterility of our products, we, as manufacturers, have employed all sorts of devices to aid us in this effort. With the help of over-zealous salesmen, we have installed so-called "sterile" rooms, precipitrons, germicidal lamps, aerosol nebulizers, and other items, some of which closely approach witchcraft. We wrap ourselves in gowns resembling Roman togas; we take off our shoes in an outer chamber as if we were entering some oriental temple, and go through a hand-cleansing ceremony anointing ourselves with bacteriostatic agents. All of this is done to avoid some invisible devils often referred to as pyrogens or contaminations.

We do this all too often without having actual proof of its value. In order to be practical about many of these procedures, about ten years ago, after visits to most of the pharmaceutical laboratories, we constructed some dust-free rooms on a slightly different pattern from that used by most manufacturers of biologics and set up a system for determining the prevalence of bacteria in these cubicles.

Room Construction.—These rooms, shown in Fig. 1, are so constructed as to permit easy cleaning, there being no table legs or furniture with the exception of the work shelves and movable chairs. All joints are rounded coves and the glass is imbedded in mastic. The doors are felt- and copper-stripped and the air inlet is by means of a Carrier Model J Air-Conditioner with oil-soaked glass wool filter. The exhaust is protected by the same filter and entrance is made through air locks. The ceilings are sloped so that if aerosol sprays are used any condensation will run off and not drip down on operators' necks. The equipment in

Figure 1.

* Presented to the joint meeting of the Scientific Section and the Biological Section of the American Drug Manufacturers Association, thirty-sixth annual meeting, Hotel Mount Washington, Bretton Woods, N. H., June 7, 1948.
Received June 24, 1948, from Biological Research Laboratories, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

the room is so arranged that five operators are required; two at filling positions and three at sealing devices (Figs. 2 and 3).

are arranged as shown in Fig. 4. At the position marked *1* under the glass shield where two fillers work, one blood agar plate and one

Figure 2.

Testing System.—We realize that occasional exposure of plates in these rooms would be of little value in determining the usefulness of the above equipment and techniques, so that a routine system of testing was instigated. We found at first that the use of plain agar plates was of little value, since most of the contamination came from the operators themselves. Blood agar plates were then tried and the counts increased enormously. Daily, four blood agar plates and two anaerobic plates (using anaerobic agar and an anaerobic lid) are exposed for fifteen minutes. These six plates

anaerobic agar plate are exposed. At positio *2* where the sealing occurs, another blood aga plate is exposed. At position *3*, an anaerobi plate as well as a blood agar plate are exposed At position *4*, on the storage table, the las blood agar plate is exposed (Fig. 5). Th standard fifteen-minute exposure does no occur at a set time daily, nor do the worker know exactly when the plates will be exposed each day. In this way a more representativ count is obtained, since the girls are forced t continue working normally in order that the can meet the daily filling schedule requirements

Next, the plates are incubated at 37.5° for forty-eight hours, and are then counted and identified, although this does not extend to serological classification. The total colonies per plate is recorded on the chart beside the position numbers, as shown in Fig. 6, and the total of all four blood agar plates is put in the space provided at the bottom. Thus, daily totals can be posted graphically, keeping the ampul workers aware of any rise in the counts and encouraging them to improve in technique. If the counts rise appreciably, it is very easy to convince the maids and porters that a better job of cleaning must be done.

By again looking at Fig. 4 it can easily be seen how a record of where each worker is located during the exposure time would be of great aid in detecting any one of them who might be disseminating large numbers of bacteria without apparent physical ill effects, yet running up

Figure 3.

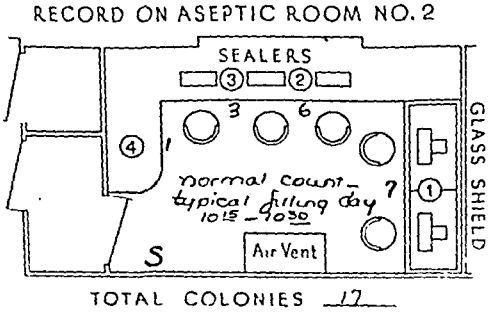


Figure 4.

the count and possibly contaminating the products. Concrete proof of the effectiveness of this method of check has been obtained several times and is illustrated by the following case.

The counts were noticed as being particularly high for about a two-week period, culminating in an extraordinary increase on the day of a sterile filling. The total count for the four aerobic blood agar plates was 164 colonies (Fig. 7). On the plate immediately adjacent to one of our operators the colonies num-

bered 90—over half the total. The other three plates showed only 23, 28, and 23 colonies. The counts proceeded normally until another alarmingly high count appeared on the day of an unsterile fill (Fig. 8). The total count was 172 for the four blood agar plates, 139 of which appeared by the worker in question. The next day's count was 145, sixty-four of which were on the plate exposed beside this operator. The count continued to be high for two more days but suddenly dropped to 9 colonies on a third day when the operator in question did not work in the filling rooms. On her return the count rose to 47, thirty-two of which

Figure 5.

Aseptic Room #2 Date 5/24/48

Sample

Plate No.	Exposure Period	Number of Colonies	Organisms
1	15 min	4	2 staph albus 2 diptheroids
2	"	3	1 staph citreus 2 staph albus
3	"	5	3 diptheroids 2 staph albus
4	"	2	2 staph albus

Notes on Reverse Side Tested by G E Longest

Figure 6.

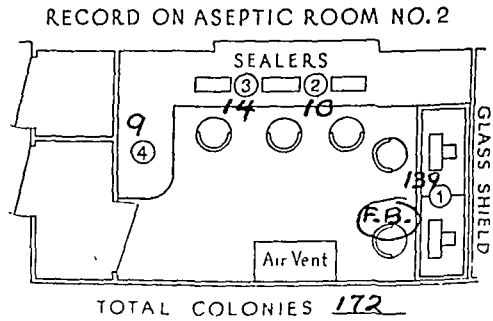


Figure 8.

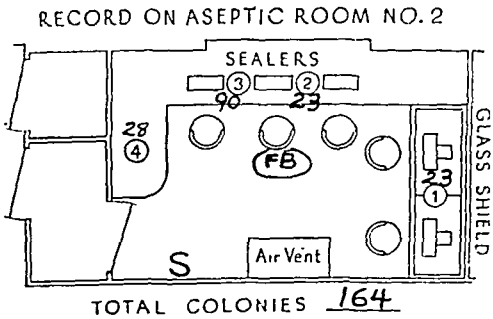


Figure 7.

occurred on the plate nearest her. The counts continued to be much above average until another day of a sterile filling when the four aerobic plates yielded 160-plus colonies (Fig. 9). The plate beside this operator was too numerous to count with a preponderance of *Staphylococcus aureus*, a decided contrast to the other three plates in the room which had only 28, 8, and 11 colonies each on them (Fig. 10). The next day's count was 88, forty-eight of which occurred on the plate beside this operator. She was removed from the filling staff, after

which the counts again dropped to normal. Throughout this time, control plates were poured under exact conditions and these remained sterile. To insure further against error, each worker was taken separately into another dust-free room where she worked for fifteen minutes, surrounded by four blood agar plates. The average total four-plate count for the worker in question was 59. Figure 11 shows the highest count obtained on her. The average for the other girls was 16. Figure 12 shows a typical count. This operator was put on penicillin therapy until such time as she could undergo a tonsillectomy. The surgeon who removed her tonsils reported them as

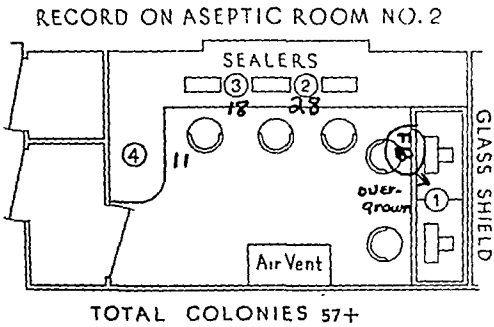


Figure 9.

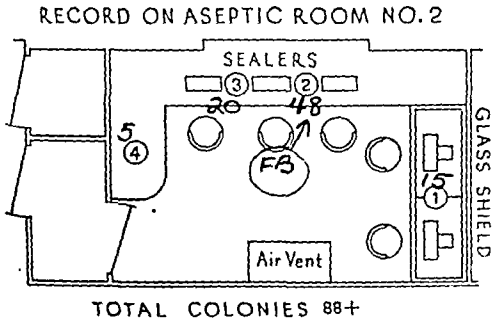
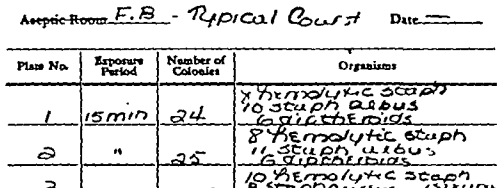


Figure 10.

being highly infected. Following this operation, her count returned to normal.

Two-year Record.—The following statistics have been compiled on the basis of the last two years' records of this daily six-plate exposure method. The average total count of the four aerobic blood agar plates for a sterile fill is 20 colonies, or 5 colonies per plate per fifteen minutes. The average total count on an unsterile fill is 27 colonies, or approximately 7 colonies per plate per fifteen minutes. (The only difference between a "sterile" and "nonsterile" fill is that no mask is worn by the workers during an "nonsterile fill" and all products ampuled by a "nonsterile fill" are autoclaved afterward. On a "sterile" fill, the workers are masked completely except for the eyes, and precautions about keeping down bacteria are more rigid.)

Considering the types of bacteria which appear on these plates, we have found that *Staphylococcus albus* (hemolytic and nonhemolytic varieties), diphtheroids, *Staphylococcus aureus*, and *Staphylococcus citreus* appear in that order of frequency. Very few Gram-positive spore-forming bacilli have been picked up. During the entire year 1946–1947 no spore formers were picked up on the plates. Molds and fungi of various types, streptococci, and

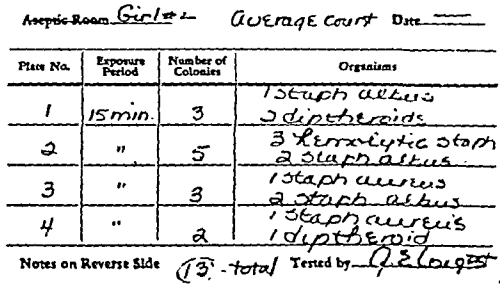


Aseptic Room F.B - Typical Count Date —

Plate No.	Exposure Period	Number of Colonies	Organisms
1	15 min	24	8 hemolytic staph 10 staph albus 6 diphtheroids
2	"	25	8 hemolytic staph 11 staph albus 6 diphtheroids
3	"	35	10 hemolytic staph 8 staph aureus 1 staph citreus 4 staph albus 7 diphth
4	"	30	1 mostly gray gr-rod 13 staph 8 hemolytic staph albus 4 staph aureus 4 diph

Notes on Reverse Side (114) total Tested by [illegible]

Figure 11.



Aseptic Room Girl #1 Average Count Date —

Plate No.	Exposure Period	Number of Colonies	Organisms
1	15 min.	3	1 staph albus 2 diptheroids
2	"	5	3 hemolytic staph 2 staph albus
3	"	3	1 staph aureus 2 staph albus
4	"	2	1 staph aureus 1 diptheroid

Notes on Reverse Side (13) total Tested by [illegible]

Figure 12.

sarcinae are found from time to time. No anaerobic organisms have been identified other than facultative organisms of the same types found on the aerobic plates.

This technique has become invaluable to us in that the average count in various seasons with different products has been in existence for so many years that it affords us an opportunity to test various new aseptic equipment and prove or disprove such things as cold cathode as opposed to hot cathode "sterilamps"; the use of glycol waxes or mopping aids, different types of aerosol spraying equipment; the use of gowns with stockinet sleeves as opposed to loose sleeves, and the value of various types of masking equipment.

SUMMARY

We have found that although the name "steri-lamps" is a misnomer, if left burning continuously, they will reduce the normal count 50% in our particular setup. This is true of both the cold and hot cathode type, although we have a slight preference for the hot cathode type since fixtures available for these seem safer and more satisfactory.

As to the gowns, we find a stockinet sleeve, wrap-around type operating gown which ties in the rear with a Hopkins-type operating hood and mask combined gives us consistently lower counts than other types.

We do not feel that at the present state the use of aerosol nebulizers is warranted.

We have found the best general check on our rooms, equipment, and technique is periodically to autoclave 8 liters of Trypticase soy broth (the best culture medium we know how to make), give it a stock number, and give it to the girls to fill into ampuls. When they have finished, place all the ampuls in the incubator and see how many show growth after seven days' incubation. This is a challenging project and, if done periodically, should build confidence or cause the manufacturer to take corrective measures immediately.

Studies on the Metabolism of 2-[α-(2-Dimethylaminoethoxy)-α-Methylbenzyl]-Pyridine Succinate (Decapryn Succinate)[1,*]

By FRED H. SNYDER, GERTRUDE R. KLAHM, and HAROLD W. WERNER

Experiments in rats and dogs, employing various dosage regimes, demonstrate urinary elimination accounts for roughly only 10 to 30 per cent of administered Decapryn Succinate. The antihistamine does not accumulate in various body tissues, and it is concluded that the bulk of administered Decapryn Succinate is destroyed in the body.

PHARMACOLOGICAL and clinical studies on a new antihistamine compound, 2-[α-(2-dimethylaminoethoxy)-α-methylbenzyl]-pyridine succinate (Decapryn Succinate), have recently been reported (1–7). The present paper deals with investigations of the fate of this compound following administration to laboratory animals.

METHODS

Animal Work.—Studies have been carried out on urinary elimination and tissue concentrations in white rats and mongrel dogs. Evaluations in rats were made following single intravenous administrations of Decapryn Succinate and following single and repeated oral administrations of this histamine antagonist. Urine was collected by housing the animals over large funnels, and the urine was separated from feces by the device described by Gross and Connell (8).

Urinary elimination and in some experiments blood levels were determined for dogs which had received Decapryn Succinate in hard gelatin capsules three times a day for thirty to fifty days. Animals were housed in the usual type of metabolism cage for urine collection.

Analytical Procedure.—The picric acid colorimetric method of Richter (9) was used for the determination of Decapryn in urine and tissues. This is a general method for aliphatic amines, and color production is not necessarily limited to unchanged Decapryn. Degradation products may thus be responsible for a portion of the color produced, but the results give important information regarding the appearance of extra amine in the urine.

Recovery experiments have demonstrated

[1] This trade name of The Wm. S. Merrell Co. will be used throughout the paper for convenience.

* Received June 22, 1948, from the Pharmacology Department, Research Laboratories, The Wm. S. Merrell Co., Cincinnati, Ohio.

that the method is accurate for the determination of Decapryn Succinate to within 3 per cent. Other studies have shown that the excreted product is stable in urine at room temperature for at least the twenty-four-hour period employed in collecting specimens.

EXPERIMENTAL RESULTS

Excretion Studies in Control Rats.—Twenty-four-hour urine samples were collected individually from 25 normal adult rats of varying age and weight and of both sexes, and samples were assayed by the colorimetric procedure. The amount of chromogenic material excreted, expressed as Decapryn Succinate, varied between 0.1 mg. and 0.2 mg./day, with an average of 0.15 mg./day. This quantity is not significant in those experiments where moderate amounts of amine are excreted, but it assumes some importance when small doses are administered or when delayed excretion is measured. Consequently, in order to avoid attaching undue significance to trace excretion, an arbitrary control value of 0.2 mg. was subtracted from each twenty-four-hour figure obtained with experimental animals.

Excretion Following Intravenous Administration to Rats.—The excretion of Decapryn following its introduction directly into the blood stream was determined in a group of 12 animals. The dose employed, 25 mg./Kg., produced some signs of toxicity, but no deaths resulted. The use of smaller doses was not feasible because the amounts excreted would have fallen too close to the control level.

As shown in the upper part of Table I, the 6 male rats excreted an average of about 8%, and the 6 female rats excreted an average of about 17% of the administered dose in twenty-four hours. This difference appears to be statistically significant.

Excretion during the second day following drug administration was very low, averaging less than 1% of the dose, and there was no significant difference between the sexes.

Excretion Following Single Oral Doses to Rats.—Three dose levels of Decapryn Succinate and a total of 56 animals were used in the studies.

Table I shows that the percentage elimination was lowest with the smallest dose (100 mg./Kg.). The highest dose given (500 mg./Kg.) was in the toxic range, and, as indicated by the standard errors in the table, variation in excretion was greater. Nevertheless, the results were generally consistent with those obtained at other dose levels.

Excretion was essentially complete within twenty-four hours after the oral administration of 100 and 250 mg./Kg., but, at 500 mg./Kg., considerable amounts appeared in the urine during the second day.

The sex difference observed with single intravenous doses was also apparent with single oral doses, especially during the first twenty-four hours following drug administration. Age and weight differences were excluded as contributing factors by comparing properly selected groups within the large group.

Excretion with Repeated Oral Doses to Rats.—Decapryn Succinate was administered orally to rats once a day for periods of four or eleven days in two different experiments employing several dose levels. It is seen from Table II that excretion tended to increase during the first few days of the experiment, but there was considerable irregular variation. In two instances, excretion remained at quite a high level on the day after the cessation of

TABLE I.—URINARY EXCRETION OF DECAPRYN FOLLOWING THE ADMINISTRATION OF SINGLE DOSES OF DECAPRYN SUCCINATE TO RATS

Route of Administration	Sex	Dose, Mg./Kg.	Decapryn Excretion in Urine: 1st 24 Hr. No. of Animals	1st 24 Hr. Mean and Standard Error, % of Dose	2nd 24 Hr. No. of Animals	2nd 24 Hr. Mean and Standard Error, % of Dose
I. V.	M	25	6	7.6 ± 1.3	6	0.9 ± 0.6
I. V.	F	25	6	16.9 ± 2.7	6	0.5 ± 0.3
Oral	M	100	10	8.1 ± 1.2	3	0.1 ± 0.1
Oral	F	100	16	17.5 ± 1.5	3	0.6 ± 0.1
Oral	M	250	8	14.4 ± 1.9	2	0.9 ± 0.5
Oral	F	250	10	23.1 ± 2.9	2	1.4 ± 1.4
Oral	M	500	6	14.3 ± 2.5	3	7.0 ± 1.7
Oral	F	500	6	24.7 ± 5.0	3	11.0 ± 0.6

TABLE II.—URINARY EXCRETION OF DECAPRYN WITH REPEATED DAILY ORAL DOSES OF DECAPRYN SUCCINATE TO FEMALE RATS

Daily Dose, Mg./Kg.	Per Cent of Daily Dose Excreted in Urine, Days: 1	2	3	4	5	6	7	8	9	10	11	12	13
100	9.3	25.2	19.3	*33.3*[a]	0.7	1.0	..	..	..	..	..	..	..
250	16.7	21.9	30.8	*32.3*	23.4	7.1	..	..	..	..	..	..	..
250	12.2	26.6	30.1	*25.7*	17.0	1.9	..	..	..	..	..	..	..
25	1.8	12.8	8.0	8.0	27.2	9.6	8.0	3.2	4.8	4.8	*3.2*	0	0
25	2.9	11.4	10.0	11.4	20.0	22.9	11.4	7.1	8.6	8.6	*8.6*	0	0
250	16.2	33.5	31.3	28.3	37.9	34.1	40.9	18.3	49.7	43.4	*24.9*	2.4	0
250	26.3	30.5	35.5	25.3	41.0	42.8	42.8	41.0	46.4	28.2	*21.7*	6.5	1.9

[a] Italicized figures indicate last day of drug administration.

drug administration, and, in one of these, an amount equivalent to 7.1% of the daily dose was excreted on the next day. In other animals, particularly on the lower doses, the urinary excretion of the amine stopped almost entirely within twenty-four hours after drug administration was discontinued.

Excretion with Repeated Oral Doses to Dogs.—Table III gives the results of studies with dogs

TABLE III.—URINARY EXCRETION OF DECAPRYN WITH REPEATED ORAL DOSES OF DECAPRYN SUCCINATE IN DOGS

No. of Animals	No. of Samples	Dose, Mg./Kg. t.i.d.	No. of Days on Test	Mean and Standard Error of Decapryn Excretion, % of Dose
4	8	3.0	36–46	18.3 ± 1.6
4	8	7.5	30–50	17.1 ± 1.5

which had received Decapryn Succinate three times a day for thirty to fifty days. The average twenty-four-hour excretion value, expressed as Decapryn Succinate, for 4 control dogs was 4.4 ± 1.3 mg. This mean value was subtracted from the figures for experimental dogs before calculating the percentage excretion.

On both of the dose levels studied, the excretion amounted to about 18% of the dose and was thus of the same order of magnitude as that found for rats receiving moderate oral doses. However, there was no difference in excretion levels of male and female dogs, and the values are combined in the table.

Identification of the Material Excreted by Rats.—Since the colorimetric method used is not specific, several studies were carried out in an attempt further to characterize the excretory product. Several batches of composite urine samples, from rats which received Decapryn Succinate, were employed as starting material. The samples were made alkaline and extracted with toluene. The toluene was then extracted with dilute hydrochloric acid, and the resulting water-clear solution was used in subsequent tests. On the basis of colorimetric assays, these extractions were performed without loss of chromogenic material.

Ultraviolet Absorption.—An aqueous sample was made alkaline and extracted with chloroform; a portion of this extract was diluted to contain the colorimetric equivalent of 4 mg. of Decapryn base/100 cc. The ultraviolet absorption curve for this solution and that for a standard solution containing 4 mg. of Decapryn base/100 cc. of chloroform were determined with a Beckman spectrophotometer. The marked similarity of these curves, shown in Fig. 1, constitutes presumptive evidence that the excreted material consists largely of unchanged Decapryn.

Isolation of the Hydrochloride.—A chloroform extract of the aqueous solution was evaporated to dryness, and the residue was taken up in petroleum ether. To this solution was added a small volume of a solution of dry hydrogen chloride in petroleum ether. The resulting white precipitate was filtered, washed with petroleum ether, and dried. This material was recrystallized three times from isopropanol. The purified product had a melting point of 160–162° (uncorr.). A known sample of Decapryn hydrochloride melted at 162–164° (uncorr.), and the melting point of a mixture of the two was 159–160° (uncorr.). This demonstrates further that Decapryn base was responsible for at least a part of the color produced in the assay.

Precipitation of the Reineckate.—The insolubility of many amine reineckates suggested an additional possibility for the study of the excreted material. The conditions used for precipitation were those described by Rennick, *et al.* (10), for the determination of the tetraethylammonium ion. A 2% solution of Reinecke salt in methanol was added to an aqueous solution of Decapryn Succinate, and precipitation was completed by refrigeration. The average weight of dry precipitate was 1.5 mg./mg. of Decapryn Succinate used; this is the theoretical value for the formation of Decapryn monoreineckate.

It was observed further that, when these precipitates were suspended in hot water, made alkaline with sodium hydroxide, and extracted with toluene,

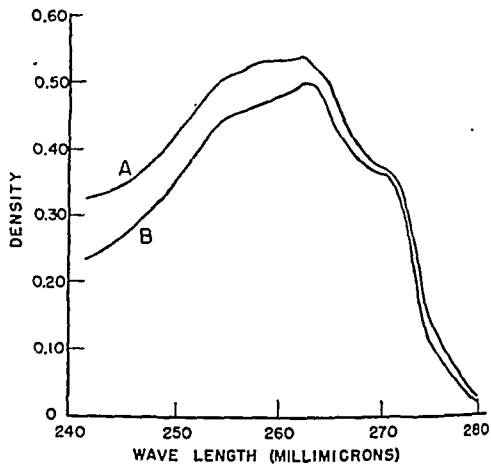


Fig. 1.—Ultraviolet absorption curves of Decapryn solutions.

Curve A: 4 mg. of Decapryn base dissolved in 100 cc. of chloroform. *Curve B:* Urine extract containing the colorimetric equivalent of 4 mg. of Decapryn base in 100 cc. of chloroform.

colorimetric assay gave reasonably satisfactory recovery (94 to 104%) of the initial quantity of Decapryn Succinate.

This procedure was consequently applied to extracts of pooled rat urine. The weights of the precipitates, converted to terms of Decapryn Succinate on the basis of monoreineckate formation, corresponded to recoveries of 95–97% of the material shown to be present by the colorimetric method.

Acetone solutions of the precipitated reineckates were prepared, and color intensities were determined in the Fisher Electrophotometer with a 525 mμ filter. The data showed that samples obtained from Decapryn Succinate and from urine extracts were identical in this respect.

Portions of the precipitates, without further purification, were subjected to elementary analysis. The results (Table IV) show only that neither substance was chemically pure.

Protection Against Lethal Effects of Histamine.—Several of the aqueous extracts of urine were in-

jected intravenously into guinea pigs and the degree of protection afforded against the lethal effects of intravenously administered histamine was determined. Concentrations of the solutions used were based on the values obtained from colorimetric assay, and it was found that an amount equivalent, colorimetrically, to 2 mg. of Decapryn Succinate/Kg. protected against as many as 7 fatal doses of histamine, while the equivalent of 16 mg./Kg. protected against as may as 60 fatal doses. It is apparent that the excreted material has a rather potent antihistaminic action.

TABLE IV.—ELEMENTAL ANALYSIS OF DECAPRYN REINECKATE PRECIPITATES

Source of Precipitate	Composition, % C	H	N	Cr
Decapryn Succinate	43.22	5.30	17.66	8.90
Urine extract	35.71	6.50	18.74	10.80
Theoretical for Decapryn Monoreineckate	42.84	4.78	19.04	8.83

Decapryn Succinate in Tissues.—Application of the colorimetric method demonstrated that no trace of chromogenic amine was present in the blood, liver, spleen, heart, lungs, kidneys, or skeletal muscle of control rats, or in the blood of control rabbits and dogs. The same tissues of rats also showed no trace of amine twenty-four hours after they received single oral doses of 100 mg. of Decapryn Succinate/Kg. Other assays demonstrated the absence of amine in the blood (*a*) of rats one hour after the oral administration of 25 mg. of Decapryn Succinate/Kg., (*b*) of rabbits one hour after the intravenous administration of 20 mg./Kg., and (*c*) of dogs thirty-six hours after the termination of an experiment in which a dose of 7.5 mg./Kg. was administered orally three times daily for about eight weeks.

SUMMARY

1. Average urinary excretion values ranged, depending on the size of the dose, between 7 and 21 per cent of the dose for male rats and between 17 and 36 per cent for females following the administration of single intravenous and oral doses of Decapryn Succinate. Excretion was virtually complete within twenty-four hours following doses of 250 mg./Kg. or less.

2. Excretion by rats tended to increase during the first few days of repeated daily oral administration, and it dropped sharply after drug administration was discontinued. Dogs receiving daily oral doses of Decapryn Succinate for prolonged periods consistently eliminated roughly 20 per cent of the daily dose in the urine, and there was no difference in excretion between the sexes.

3. No trace of amine was found in the tissues of rats twenty-four hours after the oral administration of 100 mg. of Decapryn Succinate/Kg., or in the blood under any of the conditions employed.

4. The probable identity of the excreted material and Decapryn has been established by (*a*) ultraviolet absorption studies, (*b*) isolation of Decapryn hydrochloride, (*c*) precipitation of the reineckate, and (*d*) measurement of protection against the lethal effects of histamine.

5. It is concluded that the bulk of the administered Decapryn Succinate is destroyed in the body.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

3,5-Diiodothyronine
D-Galactoascorbic acid
L-Glucoascorbic acid
Rubrophen
Hypoxanthine desoxyribose phosphoric acid
Fructose-6-phosphate
Kellin
Hydnocarpic acid
Petroselinic acid
Isopelletierine
D-Lupanine hydrochloride
Tigogenin
Betulin
Phenol sulfatase

The Antibacterial Actions of Quaternary Ammonium Sulfonamides*

By C. A. LAWRENCE and G. R. GOETCHIUS,† with the Technical Assistance of J. F. GAIN

The results of a study of the antibacterial actions of a number of quaternary ammonium sulfonamides are reported. The antibacterial actions of these compounds have been found to be due to the quaternary ammonium component in the molecule and not to the sulfonamide radical.

THE DESIRABILITY of having available a single compound which exhibits not only sulfonamide action but bactericidal, surface-acting properties as well, has been suggested by Barry and Puetzer (1). These workers implied that a compound containing a highly germicidal quaternary ammonium residue as the surface-active portion of the molecule, combined with a sulfonamide group, may be useful in nasal therapy and for other chemotherapeutic applications. The chemistry of several of these compounds, namely the cetyltrimethyl ammonium, cetylpyridinium, and laurylpyridinium salts of one or more of the commonly known sulfonamides, was described by the workers mentioned. They concluded from their studies that the compounds were highly bactericidal against *Staphylococcus aureus* and *Escherichia coli, in vitro*. In some instances, the compounds were more effective than the corresponding ammonium components contained in the molecule.

Studies in our laboratories have indicated several other quaternary ammonium sulfonamides are likewise effective in high dilutions in destroying a variety of pathogenic bacteria. Unlike the compounds described by Barry and Puetzer in which the sulfonamide portion of the molecule is the anion, our studies involved the use of compounds in which the sulfonamide radical is part of the cation. The difference in the structural configuration of the two types of quaternary ammonium sulfonamides, as well as the compounds used in the present study, are shown in Figs. 1 and 2. The chemistry of the latter will be described elsewhere by Dr. E. D. Homiller of these laboratories.

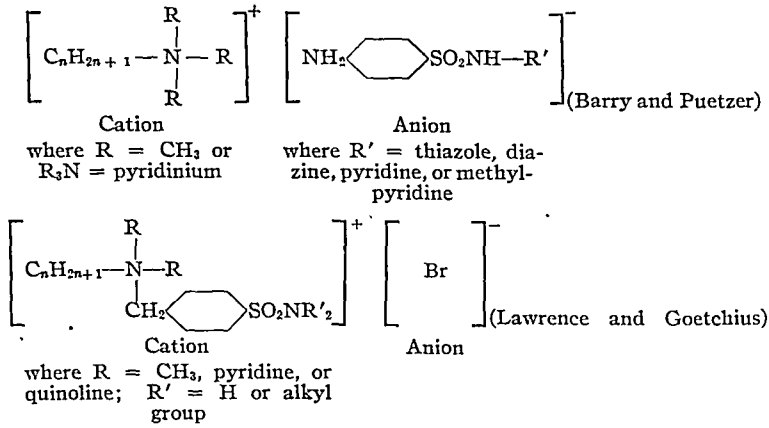

Fig. 1.—Structural formulae of the two types of quaternary ammonium sulfonamides.

EXPERIMENTAL

Phenol Coefficient.—The Food and Drug Administration (F. D. A.) method for determining phenol coefficients (2) was one means of evaluating the quaternary ammonium sulfonamides. *S. aureus* (No. 209) and *Eberthella typhosa* (Hopkins strain) were used as the test organisms. The seeding tubes containing the dilutions of the compounds were placed in a water bath at 20° for five minutes, or

* Received March 26, 1948, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.
† Present address: Rohm and Haas, Philadelphia, Pa.

until the temperature of the bath was reached, before the addition of the test culture. The tubes of F. D. A. broth containing transplants from the seeding tubes were incubated at 37° for forty-eight hours and observed for the presence or absence of visible growth. Tubes showing no growth were tested for bacteriostasis by transferring a quantity (three 4-mm. loopfuls) of the broth mixture to additional tubes containing sterile broth, as outlined in the F. D. A. procedure. The phenol coefficient values on duplicate tests and the highest dilution of the quaternary ammonium sulfonamide which destroyed the test organisms in ten but not in five minutes are given in Table I.

The results of this study show the most active compounds are No. 1 (4-sulfonamidobenzyltetradecyldimethylammonium bromide) and No. 2 (4-dodecylsulfonamidobenzylpyridinium bromide). Identical phenol coefficient values were obtained on the latter salts, namely 233 and 267, against *S. aureus* and *E. typhosa*, respectively. Considerably lower phenol coefficient values were obtained on compound No. 4 (4-dodecylsulfonamidobenzyltrimethylammonium bromide) while the remaining quaternary ammonium sulfonamides failed to show evidence of a germicidal action in the highest concentration tested (1:1000 or 0.1%).

TABLE I.—PHENOL COEFFICIENTS ON QUATERNARY AMMONIUM SULFONAMIDES

	S. aureus		*E. typhosa*	
Compound	Phenol Coefficient	Limiting Dilution Effective—10 Min.	Phenol Coefficient	Limiting Dilution Effective—10 Min.
1	233	12,000	267	24,000
2	233	12,000	267	24,000
3	<16[a]	<1,000	<11	<1,000
4	83	5,000	50	5,000
5	<16	<1,000	<11	<1,000
6	<16	<1,000	<11	<1,000

[a] <16 = No phenol coefficient value obtainable since highest concentration tested (1:1000) failed to destroy the test organism in ten minutes.

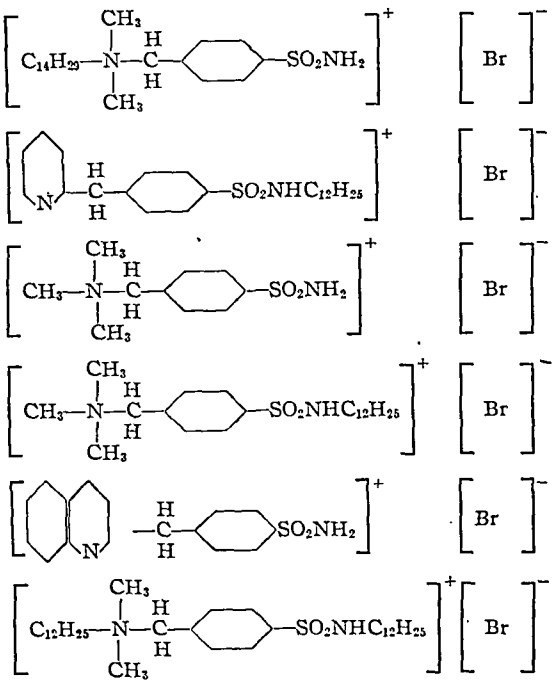

Fig. 2.—Structural formulae of quaternary ammonium sulfonamides.

Bactericidal and Bacteriostatic Tests.—The compounds were tested against a variety of bacteria in the presence of a fluid medium by dissolving them directly in broth in a 1:1000 concentration. From this initial concentration, serial dilutions were made in broth up to and including 1:16,384,000. The compounds were all completely soluble in broth in the effective dilutions with the exception of No. 6 (4 - dodecylsulfonamidobenzyldimethyldodecylammonium bromide) which contained some undissolved material in the higher concentrations. The drug-broth solutions were distributed in 5-ml. quantities in test tubes and autoclaved at ten pounds for ten minutes. The inoculum consisted of one 4-mm. loopful of a twenty-four-hour broth culture of the test organism. A number of Gram-negative bacilli representative of the colon-typhoid-dysentery group were tested in the presence of beef extract broth (F. D. A.). The latter medium was also used for *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *S. aureus*. Veal infusion-glucose broth containing 1% normal horse serum was used in tests in which Types I, II, and III pneumococci and *Streptococcus pyogenes* (C-203) were studied. This same medium, but without serum, was selected for studying the other streptococci. The series was completed with tests on the *Brucella* in tryptose-phosphate broth containing 0.1% agar, and four anaerobic clostridia in fluid thioglycollate medium.

The inoculated media were incubated at 37° and

observed for the presence of visible growth at the end of twenty-four, forty-eight, and seventy-two hours. Failure of growth to appear, or to be less than half that in the drug-free control, after the initial twenty-four-hour incubation period was considered evidence of bacteriostasis. Tubes showing no growth at the end of seventy-two hours were tested for bactericidal action by transferring three 4-mm. loopfuls of the test mixture to a second tube of liquid medium. Lack of growth in the subculture tubes following seventy-two hours' incubation was taken as evidence of a bactericidal action of the compound upon the test organisms in the original tube.

The high antibacterial activity of compounds 1 and 2 is again evident from the data presented in Table II. In general, concentrations of 1:64,000 to 1:256,000 of the compounds in nutrient media proved to be bacteriostatic to the various Gram-positive cocci. In many instances the bactericidal and bacteriostatic dilutions were found to be identical.

Of the four strains of clostridia, *Cl. histolyticum* was found to be the most resistant organism to the lethal action of the quaternary ammonium compounds. Number 1 was found to be completely ineffective against the organism, whereas concentrations of 1:4000 to 1:16,000 of some of the other compounds were required to inhibit its growth.

Inspection of the data (Table II) will, furthermore, reveal compound 4 is somewhat less active than Nos. 1 and 2 against most of the organisms studied. Also, while No. 6 is entirely ineffective as a germicidal agent against all the Gram-negative bacteria, it compared in activity with compound 4 against many of the Gram-positive cocci. Although not indicated in the table, compounds 3 (4-sulfonamidobenzyltrimethylammonium bromide) and 5 (4-sulfonamidobenzylquinolinium bromide) were found to be entirely inactive to all of the organisms studied.

Surface tension.—Gershenfeld and Milanick (3), Baker, Harrison, and Miller (4), and Rawlings, Sweet, and Joslyn (5) have shown that while many of the more effective cationic detergents have low surface tension, some of the less active ones are equally surface active. Furthermore, in many cases the anionic compounds of feeble germicidal action have an effect on surface tension comparable to the most active cationic detergent.

In the present study, the surface tensions of 0.01% concentrations of the quaternary ammonium sulfonamides were determined by the "ring method" with a du Nouy tensiometer. Readings were made soon after the solutions were prepared and again after allowing them to stand at room temperature for twenty-four hours. The values, corrected for temperature, are given in Table III. Included in

TABLE II.—LIMITING DILUTIONS OF QUATERNARY AMMONIUM SULFONAMIDES SHOWING ANTIBACTERIAL ACTIVITY

Organism	Compound 1 Bs	Compound 1 Bc	Compound 2 Bs	Compound 2 Bc	Compound 4 Bs	Compound 4 Bc	Compound 6 Bs	Compound 6 Bc
Pneumococcus Type I	256	64	512	256	128	32	1024	32
Pneumococcus Type II	64	32	256	128	32	32	64	16
Pneumococcus Type III	64	64	256	256	64	64	256	256
Streptococcus pyogenes (C203)	512	256	512	128	256	64	1024	64
Streptococcus viridans	1024	512	4096	2048	512	256	256	16
Streptococcus fecalis	128	64	512	16	64	16	128	2
Streptococcus mitis	256	256	1024	1024	128	64	256	2
Staphylococcus aureus	512	128	512	256	512	64	128	<1
Pseudomonas aeruginosa	<1	<1	1	<1	1	<1	<1	<1
Escherichia coli	64	32	64	16	32	16	<1	<1
Eberthella typhosa	128	64	256	64	32	16	<1	<1
Salmonella paratyphi A	128	32	64	8	16	8	<1	<1
Shigella paradysenteriae Sonne	128	64	64	64	64	32	<1	<1
Vibrio cholerae	128	64	256	128	128	1	<1	<1
Brucella abortus	256	32	512	16	64	32	16	<1
Brucella melitensis	512	64	64	32	256	64	4	<1
Brucella suis	8	4	8	8	32	8	4	<1
Clostridium welchii	128	32	128	64	64	32	8	2
Clostridium tetani	128	64	128	64	256	64	8	2
Clostridium histolyticum	<1	<1	16	4	16	4	8	<1
Clostridium oedematiens	32	16	32	16	32	16	8	8

Bs = Bacteriostatic.
Bc = Bactericidal.
All values given represent dilutions in thousands, i.e., 256 = 256,000, etc.
Figures preceded by sign < indicate the highest concentration tested failed either to kill and, or, inhibit the growth of the test organism.

With but one exception, the Gram-negative bacilli were found to be destroyed by relatively low concentrations of compounds 1 and 2. In most instances, the bacteriostatic dilutions were but one tube removed in the serial dilutions showing a bactericidal effect on the same organism. Both compounds, as well as Nos. 4 and 6, were found to have little, if any, effect on the culture of *Ps. aeruginosa*.

A definite degree of resistance to the antibacterial actions of the quaternary ammonium sulfonamides was exhibited by *Brucella suis*. The *Br. abortus* and *Br. melitensis* strains, on the other hand, were inhibited in their growth and destroyed by relatively high dilutions of compounds 1, 2, and 4.

the table are surface-tension readings on 0.01% aqueous and broth solutions of compounds 3 and 5 in the presence of 0.1% of a nonionic detergent.[1]

From the data (Table III), it will be noted that compounds 3 and 5, which were ineffective as antibacterial agents, were correspondingly inactive as surface-tension depressants in water and nutrient broth. The low surface-tension environment is not the only factor responsible for the high germicidal activity of several of the other quaternary ammonium sulfonamides as evidenced from the surface-tension values obtained on compound 6. This compound is the most active surface-tension depressant in the series studied, yet it failed to give a phenol coefficient value against *S. aureus* and *E. typhosa* (Table I) and was, furthermore, less active than compounds 1, 2, and 4 when tested in the presence of a nutrient medium (Table II).

TABLE III.—SURFACE TENSIONS OF AQUEOUS AND BROTH SOLUTIONS OF QUATERNARY AMMONIUM SULFONAMIDES

Compound Number	Surface Tension of 0.01% Solutions: Distilled Water	Surface Tension of 0.01% Solutions: F. D. A. Broth
1	59.7	42.7
2	56.8	38.4
3	75.2	53.4
4	58.5	38.9
5	75.3	53.7
6	31.4	32.2
3 (+ 0.1% nonionic detergent)	35.8	36.4
5 (+ 0.1% nonionic detergent)	37.0	37.6
Controls		
Distilled water	75.3	..
F. D. A. Broth	..	53.7
0.1% nonionic detergent	34.5	..

The relationship of surface tension to the antibacterial action was further investigated in the following manner: The nonionic detergent of low surface tension, which in itself was devoid of any demonstrable antibacterial action, was incorporated in a medium containing 0.01% of compounds 3 and 5. The resulting solutions, with surface tensions of 36.4 and 37.6, respectively (Table III), were incapable of inhibiting the growth of test organisms. Our studies, furthermore, indicated the nonionic detergent and the active quaternary ammonium sulfonamides were compatible with respect to the antibacterial actions of the latter.

Germicide Inactivation.—Since the quaternary ammonium sulfonamides contain two possible antibacterial components, a quaternary ammonium and a sulfonamide group, it appeared desirable to determine whether the bactericidal and bacteriostatic activities could be attributed to all, or part, of one of these components in the molecule. A test was designed, therefore, to include a suitable quaternary inactivator and a sulfonamide inactivator in the medium in which the more active quaternary ammonium sulfonamides were tested against a representative culture of the Gram-positive, as well as the Gram-negative, group of bacteria.

Compounds 1 and 2 were dissolved primarily in distilled water to a concentration of 1:200. Serial dilutions were prepared in F. D. A. broth in a range from 1:2000 up to and including 1:256,000. To 4 ml. of the drug dilution was added either 1 ml. of 1:5000 para-aminobenzoic acid (sulfonamide inactivator), 1 ml. of 1% Suramin Sodium (6), U. S. P. XIII (quaternary ammonium inactivator), or 1 ml. of distilled water (control). One series of tubes was inoculated with a 4-mm. loopful of a twenty-four-hour broth culture of *S. aureus* while another series received an inoculum of *E. typhosa*. The tubes were incubated at 37° for seventy-two hours and the presence or absence of growth recorded at this time. Tubes showing no growth at the seventy-two-hour period were tested for bacteriostasis by subculturing three 4-mm. loopfuls of the test suspension to 10 ml. of plain F. D. A. broth, as well as to F. D. A. broth containing Suramin Sodium. The latter compound was also added directly to the original medication tubes showing no visible growth at the seventy-two-hour observation period. Lack of growth in the subculture tubes, following seventy-two hours' incubation, was considered evidence of a bactericidal action of the quaternary ammonium sulfonamides upon the test organisms in the original medication tubes. Since the results of the test on the two quaternary ammonium compounds were comparable, the data obtained on only one of these are presented in Table IV.

It is evident from the data given in the table that

TABLE IV.—INACTIVATION OF QUATERNARY AMMONIUM SULFONAMIDES

Dilution	*S. aureus* Control 1[a]	2	3	4	*S. aureus* PABA 1	2	3	4	*S. aureus* Suramin 1	2	3	4	*E. typhosa* Control 1	2	3	4	*E. typhosa* PABA 1	2	3	4	*E. typhosa* Suramin 1	2	3	4
1:2,000	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—	—	—	—	+	+	+	+
1:4,000	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—	—	—	—	+	+	+	+
1:8,000	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—	—	—	—	+	+	+	+
1:16,000	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—	—	—	—	+	+	+	+
1:32,000	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—	—	—	—	+	+	+	+
1:64,000	—	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:128,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:256,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] 1 = Original medication tube; 2 = Medication tube + Suramin Sodium added at seventy-two-hour period; 3 = F D A broth subculture tube; 4 = F D A broth + Suramin Sodium subculture tube; — = No visible growth; + = Growth.

[1] One of a series of viscous, water-soluble nonionic agents which are described as long-chain fatty acid esters containing multiple ether linkages, known as Intral 224, a Synthetic Chemical, Inc., product.

the activity of the quaternary ammonium sulfonamides can be attributed to the quaternary component in the molecule. The antibacterial activity could be completely neutralized by Suramin Sodium, whereas para-aminobenzoic acid failed to have any adverse action on the germicidal action of the compounds in the dilutions tested.

The data furthermore reveal that the quaternary ammonium compounds possess a definite bactericidal action in relatively high dilutions, since in most instances the organisms could not be "revived" upon the addition of Suramin Sodium to the original "control" and "PABA" series at the seventy-two-hour period.

SUMMARY

Certain salient features to be noted from a comparison of the structural configuration to the antibacterial activities of several quaternary ammonium sulfonamides may be listed as follows:

1. The addition of a long-chain alkyl group on the quaternary nitrogen of 4-sulfonamidobenzyltrimethylammonium bromide will result in a compound with high antibacterial and surface-tension depressant actions.

2. Substitution of a long-chain alkyl group on the sulfonamide nitrogen of 4-sulfonamidobenzyltrimethylammonium bromide will also result in a compound with considerable activity.

3. A compound containing a long-chain alkyl group on both the quaternary and sulfonamide nitrogens of 4-sulfonamidobenzyltrimethylammonium bromide has a low surface tension, but its germicidal effects are less marked than those of the single alkyl substituted derivatives.

4. The antibacterial actions of the quaternary ammonium compounds described in this study are due to the quaternary ammonium component in the molecule and not the sulfonamide radical.

Zienty (7), in a recent publication appearing after the present study had been completed, described a large series of quaternary ammonium salts of pyridine-3-carboxamides and N'-substituted pyridine-3-sulfonamides. A number of the latter compounds compared favorably with the germicidal activity of benzalkonium chloride U. S. P. XIII.

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Studies on Alcohol-Soluble Fungistatic and Fungicidal Compounds. III. Evaluation of the Antifungal Properties of Quinones and Quinolines*

By KURT A. OSTER and MILTON J. GOLDEN†

Ten commonly used antifungal compounds have been found to possess only relatively weak fungistatic and fungicidal properties. In a search for more potent therapeutic substances fifty-five chemicals belonging to the quinone and quinoline series were subjected to a preliminary screening test, and twelve of these were subsequently subjected to more intensive *in vitro* studies. Four compounds of the series were found to possess outstanding antifungal properties. The potency of one chemical was as much as seventy-seven times greater than that of the average substances now in therapeutic usage.

In two previous publications (1, 2) the currently used laboratory methods for evaluating fungistatic and fungicidal compounds were subjected to a critical study. The necessity for modifying these tests was proved in order for them to be regarded as significant and to permit comparison of true potency of antifungal substances. A new expression of fungistasis, the activity coefficient, was introduced at this time and the fungicidal tests were made more stringent. Use of these methods, thus modified and improved, demonstrated that ten compounds now popularly used in the treatment of dermatophytosis possess only relatively weak fungistatic and fungicidal properties.

This finding prompted the search for more specific antifungal compounds, the potencies of which could be measured by the improved laboratory test methods and which, after screening out the less active substances, could be subjected to more intensive laboratory studies and finally to clinical trials. This investigation is concerned with the laboratory testing of compounds belonging to the quinone and quinoline series, some of which are known to exert certain antifungal action.

Reports that certain quinones are bactericidal have been forthcoming for the last forty years (3). The fungicidal properties of quinones was the subject of a patent issued in 1926 to Brodersen and Ext (4). Tetrachloro-*p*-benzoquinone was patented as a fungicide by Ter Horst (5). The same investigator described the fungicidal properties of 2,3-dichloro-1,4-naphthoquinone for agricultural and textile uses in 1943 (5). Gonzalez (6) reported the inhibitory effect of quinones on the growth of *Penicillium notatum*. Colwell and McCall (7) investigated the mechanism of bacterial and fungus growth inhibition by 2-methyl-1,4-naphthoquinone, suggesting that its antibacterial and antifungal effects are attained by blocking sulfhydryl-containing enzymes or growth metabolites, leaving open, however, the possibility of different mechanisms.

In 1947, Ball, Anfinsen, and Cooper (8), investigating the inhibition of respiratory processes by naphthoquinones, found that the activity of these compounds depends upon the nature of the alkyl group and suggested that the inhibitory action results from a combination of the naphthoquinones with an unknown enzyme which mediates the reaction between cytochrome *c* and cytochrome *b*. Hoffman-Ostenhof (9) stated that the antibiotic action of quinones is due not to a single reaction, but to a complex mechanism. The mode of action proposed by Colwell and McCall (7) would be only one of the means by which the quinones exert their effects and would not be the dominant one in most cases.

8-Hydroxyquinoline, first introduced in Germany in 1895 as an "antiseptic and disinfectant," has long been used as a bactericidal and fungicidal agent in many medical preparations. Its fungistatic action was explained by Zentmyer (10) as being due to "the phenomenon of forming inner complex salts with metal ions and thus rendering them unavailable to microorganisms." The same hypothesis is under investigation by Albert and his collaborators (11). Rigler and Greathouse (12) studied the fungicidal potencies of quinoline homologs and derivatives against *Phymatotrichum omnivorum*, a fungus causing cotton root disease, and found 8-hydroxyquinoline to be over six hundred times more potent than the 2-isomer.

The antifungal action of quinones and quinolines has not yet been studied intensively on that group of fungi which causes human dermatophytosis, as far as our study of the pertinent literature has revealed.

* Received February 2, 1948, from the Research Laboratories, McKesson and Robbins, Inc., Bridgeport, Conn. Presented in part before the Division of Biological Chemistry of the American Chemical Society at its semiannual meeting Atlantic City, N. J. April 1946.

† The authors wish to express their thanks to Dr. Louis F. Fieser, Harvard University, Dr. Nelson J. Leonard, University of Illinois, and Dr. Byron Riegel, Northwestern University, for their generous supply of chemicals used in this study. Miss Irene Pak rendered valuable technical assistance throughout the entire investigation.

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This finding prompted the search for more specific antifungal compounds, the potencies of which could be measured by the improved laboratory test methods and which, after screening out the less active substances, could be subjected to more intensive laboratory studies and finally to clinical trials. This investigation is concerned with the laboratory testing of compounds belonging to the quinone and quinoline series, some of which are known to exert certain antifungal action.

Reports that certain quinones are bactericidal have been forthcoming for the last forty years (3). The fungicidal properties of quinones was the subject of a patent issued in 1926 to Brodersen and Ext (4). Tetrachloro-*p*-benzoquinone was patented as a fungicide by Ter Horst (5). The same investigator described the fungicidal properties of 2,3-dichloro-1,4-naphthoquinone for agricultural and textile uses in 1943 (5). Gonzalez (6) reported the inhibitory effect of quinones on the growth of *Penicillium notatum*. Colwell and McCall (7) investigated the mechanism of bacterial and fungus growth inhibition by 2-methyl-1,4-naphthoquinone, suggesting that its antibacterial and antifungal effects are attained by blocking sulfhydryl-containing enzymes or growth metabolites, leaving open, however, the possibility of different mechanisms.

In 1947, Ball, Anfinsen, and Cooper (8), investigating the inhibition of respiratory processes by naphthoquinones, found that the activity of these compounds depends upon the nature of the alkyl group and suggested that the inhibitory action results from a combination of the naphthoquinones with an unknown enzyme which mediates the reaction between cytochrome *c* and cytochrome *b*. Hoffman-Ostenhof (9) stated that the antibiotic action of quinones is due not to a single reaction, but to a complex mechanism. The mode of action proposed by Colwell and McCall (7) would be only one of the means by which the quinones exert their effects and would not be the dominant one in most cases.

8-Hydroxyquinoline, first introduced in Germany in 1895 as an "antiseptic and disinfectant," has long been used as a bactericidal and fungicidal agent in many medical preparations. Its fungistatic action was explained by Zentmyer (10) as being due to "the phenomenon of forming inner complex salts with metal ions and thus rendering them unavailable to microorganisms." The same hypothesis is under investigation by Albert and his collaborators (11). Rigler and Greathouse (12) studied the fungicidal potencies of quinoline homologs and derivatives against *Phymatotrichum omnivorum*, a fungus causing cotton root disease, and found 8-hydroxyquinoline to be over six hundred times more potent than the 2-isomer.

The antifungal action of quinones and quinolines has not yet been studied intensively on that group of fungi which causes human dermatophytosis, as far as our study of the pertinent literature has revealed.

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† The authors wish to express their thanks to Dr. Louis F. Fieser, Harvard University, Dr. Nelson J. Leonard, University of Illinois, and Dr. Byron Riegel, Northwestern University, for their generous supply of chemicals used in this study. Miss Irene Pak rendered valuable technical assistance throughout the entire investigation.

METHODS

Laboratory methods employed in the evaluating of the quinone and quinoline compounds have been described in detail in two previous publications (1, 2). For determining fungistasis, methods described by Burlingame and Reddish (13) and by Schamberg and Kolmer (14) were utilized. Fungicidal properties of the chemicals were measured by a modification (2) of the original Burlingame and Reddish technique. Ten-day-old cultures of *Trichophyton mentagrophytes* (No. 640 Emmons) served as test organism throughout the entire investigation. All substances were dissolved in 95% ethyl alcohol, if their solubilities so permitted. A few compounds, insoluble in alcohol in the desired concentration, were dissolved in acetone. The reported results represent the average value of at least three separate determinations. All cultures were incubated at 28° C ± 1°.

EXPERIMENTAL

Eleven derivatives of benzoquinone, twenty derivatives of naphthoquinone and twenty-four quinoline derivatives were evaluated. These fifty-five compounds were first subjected to a screening test for the purpose of eliminating those which were weak in fungistatic activity. The screening procedure consisted of measuring the radius segment of the cleared zone of growth inhibition around the solution cup according to the method of Burlingame and Reddish (13), using in each case a 0.1% solution of the test compound in 95% ethyl alcohol for the evaluation. Tables I, II, and III list the compounds tested and the results obtained in millimeters clearance on seeded Sabouraud's maltose agar plates.

Dosage-response curves were obtained with various concentrations of the twelve selected test substances, using the Burlingame and Reddish test method (Fig. 1). Activity coefficients of the compounds were calculated from these curves by dividing the interpolated concentration which effects a 5-mm. radius segment of clearance into the figure one, as was suggested by Oster and Golden (1).

The same group of twelve compounds was also tested for fungistatic activity by the method of Schamberg and Kolmer (14), employing the procedure of incorporating various dilutions of the test compound in Sabouraud's maltose agar. Table IV lists the maximum dilutions effecting complete fungistasis with this technique and the activity coefficients calculated from the dosage-response curves of Fig. 1.

Fungicidal action of the investigated compounds was tested according to the authors' modification of the Burlingame and Reddish technique (2). The severe criterion of a one-minute contact time of the test substance with the test fungus was again used in this evaluation. The age of the culture was at least ten days, and the medium contained 10% serum. Table V lists the minimum fungicidal concentrations of ten of the twelve investigated compounds. To arrive at these values at least two out of three determinations for each substance had to be proved fungicidal.

RESULTS

Of the eleven representatives of the benzoquinone group listed in Table I, two compounds, *p*-xyloquinone and *p*-thymoquinone, with methyl substitutions in the 2 position and methyl and isopropyl groups, respectively, in the 5 position, exhibited highly fungistatic properties. A single methyl group in the 2 position, as in the case of *p*-toluquinone,

TABLE I.—INFLUENCE OF VARIOUS SIDE GROUPS ON THE FUNGISTATIC ACTIVITIES OF ELEVEN BENZOQUINONES (CONCENTRATION 0.1%, SOLVENT 95% ETHYL ALCOHOL)

Compound	Positions on the Ring						Clearance[a]
	1	2	3	4	5	6	Mm.
p-Quinone	O	H	H	O	H	H	6
Hydroquinone	OH	H	H	OH	H	H	None
p-Toluquinone	O	CH_3	H	O	H	H	11
Toluhydroquinone	OH	CH_3	H	OH	H	H	4
p-Phenylquinone	0	C_6H_5	H	O	H	H	8
p-Xyloquinone	O	CH_3	H	O	CH_3	H	19
p-Thymoquinone	O	CH_3	H	O	CH(CH_3)CH_3	H	18
2,5-Ditertiary butyl quinone	O	C(CH_3)$_2$—CH_3	H	O	C(CH_3)$_2$—CH_3	H	4
Rapanone	O	$(CH_2)_{12}CH_3$	OH	O	H	OH	None
2,6-Dichloroquinone	O	Cl	H	O	H	Cl	3
Chloranil (dissolved in acetone)	O	Cl	Cl	O	Cl	Cl	4

[a] Cleared zone of growth inhibition around solution cup in agar plate, measured in millimeters as radius segment.

Twelve compounds of the entire series of fifty-five were selected for further study. Of these twelve, six were chosen for their demonstrably high fungistatic activity and six for their structural nature in the hope of gaining information as to the mechanism of the strong antifungal action of the first six compounds.

showed less activity. Unsubstituted *p*-quinone exhibited low activity; as hydroquinone, its reduced form, it showed no activity whatever at the test concentration. The activity of *p*-toluquinone was also curtailed by reduction to the corresponding hydroquinone. Considering *p*-quinone as the basic value, a phenyl group in the 2 position slightly in-

TABLE II.—INFLUENCE OF VARIOUS SIDE GROUPS ON THE FUNGISTATIC ACTIVITIES OF TWENTY NAPHTHOQUINONES (CONCENTRATION 0.1%, SOLVENT 95% ETHYL ALCOHOL)

Compound	Positions on the Ring 1	2	3	4	5	6	7	8	Clearance,[a] Mm.
1,4-Naphthoquinone	O	H	H	O	H	H	H	H	5
2-Methyl-1,4-naphthoquinone	O	CH_3	H	O	H	H	H	H	17
2-Amino-1,4-naphthoquinone	O	NH_2	H	O	H	H	H	H	12
2-Chloro-1,4-naphthoquinone	O	Cl	H	O	H	H	H	H	12
2-Phenyl-1,4-naphthoquinone	O	C_6H_5	H	O	H	H	H	H	7
2-Isoheptyl-1,4-naphthoquinone	O	$(CH_2)_4C(CH_3)_2$	H	O	H	H	H	H	4
2-Sulfanilamino-1,4-naphthoquinone	O	$NHSH_2C_6H_4NH_2$	H	O	H	H	H	H	None
2,3-Dichloro-1,4-naphthoquinone	O	Cl	Cl	O	H	H	H	H	3
2-Isoamyl-3-hydroxy-1,4-naphthoquinone	O	$(CH_2)_2CH(CH_3)_2$	OH	O	H	H	H	H	6
M266[b]	O	S	OH	O	H	H	H	H	8.5
M1916[b]	O	$(CH_2)_3$—S	OH	O	H	H	H	H	None
M2279[b]	O	$(CH_2)_3$—H H	OH	O	H	H	H	H	None
M2309[b]	O	$(CH_2)_3$—O	OH	O	H	H	H	H	None
M2892[b]	O	$(CH_2)_8CH$—CH_2	OH	O	H	H	H	H	3
2-Methyl-3-hydroxy-1,4-naphthoquinone (phthiocol)	O	CH_3	OH	O	H	H	H	H	5.5
2-Methyl-3-phytyl-1,4-naphthoquinone	O	CH_3	Phytyl	O	H	H	H	H	None
2-Amino-1,4-naphthoquinonimine hydrochloride	O	NH_2	H	NHHCl	H	H	H	H	20
5,8-Dihydroxy-1,4-naphthoquinone	O	H	H	O	OH	H	H	OH	8.8
4-Amino-1,2-naphthoquinone	O	O	H	NH_2	H	H	H	H	None
4-*p*-Sulfanilino-1,2-naphthoquinone	O	O	H	NH—C_6H_4—SO_3Na	H	H	H	H	None

[a] Cleared zone of growth inhibition around solution cup in agar plate, measured in millimeters as radius segment.
[b] The designation of these compounds from Fieser, L. F., and co-workers, in press.

creased the activity, whereas chlorine substitutions apparently decreased its fungistatic effect under test conditions. Longer side chains, as the butyl or the tridecyl substitutions, either diminished or abolished the fungistatic activity of *p*-quinone.

Similar conditions prevailed with the twenty investigated compounds of the naphthoquinone series (Table II). A methyl group in the 2 position enhanced the fungistatic activity of 1,4-naphthoquinone approximately three and one-half times. An amino group or chlorine in the same position also augmented this effect but not as greatly as the methyl substitution. A phenyl group in the 2 position exhibited only a slight positive influence on the basic fungistatic value of 1,4-naphthoquinone.

The potentiating influence of the methyl-substituted 2 carbon seemed to be almost completely curtailed by the introduction of a hydroxyl group in the 3 position and abolished by a phytyl chain at the same location. The introduction of chlorine to the ring (2,3-dichloro-1,4-naphthoquinone) appeared to diminish the fungistatic activity of 1,4-naphthoquinone. (Unfortunately, the monochloro-substituted compound was not available to us.) This situation disagrees completely with that of the plant fungicides reported by Ter Horst (5), where the introduction of chlorine atoms exerts a positive influence on the demonstrable fungistatic activity.

Various other groupings on the 2 position of naphthoquinone do not contribute much information. However, it may be said that occupation of the 3 position by a hydroxyl group slightly increased the basic fungistatic activity of 1,4-naphthoquinone in one instance (M266), slightly decreased this activity in one instance (M2892 or N-decylonyl-3-hydroxy-1,4-naphthoquinone), and caused no appreciable change with two other compounds (2-isoamyl-3-hydroxy-1,4-naphthoquinone and 2-methyl-3-hydroxy-1,4-naphthoquinone). This is in contrast with the suggestion of Colwell and McCall (7) that naphthoquinones require a free 3 positi to react with sulfhydryl compounds in enzyme sy tems essential to the life of the fungus.

Of considerable theoretical interest is the ou standing action of 2-amino-1,4-naphthoquinonimi hydrochloride, the fungistatic value of which su passed those of all the other compounds tested wi this method. The activity of the only 5,8-subs tuted compound in this series, 5,8-dihydroxy-1, naphthoquinone, was only slightly greater than th of 1,4-naphthoquinone itself. The two investigat orthoquinones, 1,2-naphthoquinone and 4-*p*-sulfa lino-1,2-naphthoquinone, exhibited no activity.

Of the entire group of twenty-four quinoli derivatives (Table III), only three compoun showed a fungistatic response at the concentrati

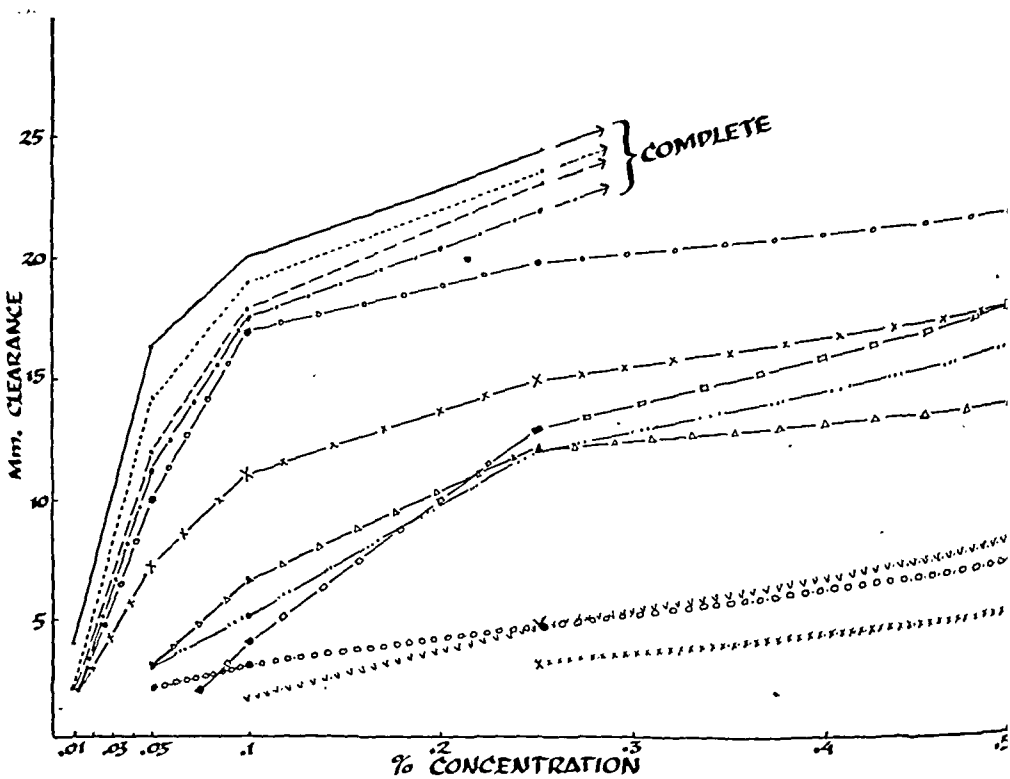

Fig. 1.—Dosage and fungistatic response curves of *T. mentagrophytes* (No. 640 Emmons) with increas ing concentrations of various quinones and 8-hydroxyquinoline. ——, 2-amino-1,4-naphthoquinonimin HCl; ------, xyloquinone; ----, thymoquinone; ·——·, 8-hydroxyquinoline benzoate; o—o—o, 2 methyl-1,4-naphthoquinone; x—x—x, toluquinone; △—△—△, quinone; ···——···, 1,4-naphthoquinone □—□—□, toluhydroquinone; o·o·o·o·o, 2,3-dichloro-1,4-naphthoquinone; v·v·v·v·v·, 2,6-dichloroquinone x·x·x·x·x·x·, hydroquinone.

TABLE III.—INFLUENCE OF VARIOUS SIDE GROUPS ON THE FUNGISTATIC ACTIVITIES OF 24 QUINOLINES (CONC. 0.1%, SOLVENT 95% ALCOHOL)

Compound	Clearance,[a] Mm.
8-Hydroxyquinoline	17.5
2,6-Dimethylquinoline	None
4,8-Dimethyl-2-hydroxyquinoline	None
3-Cyclohexyl-2,4-dihydroxyquinoline	None
7-Methoxy-4-hydroxyquinoline	None
7-Methoxy-2-methyl-4-hydroxyquinoline	None
3-Bromo-4-hydroxyquinoline	None
6-Methoxy-8-nitro-4-hydroxyquinoline	None
6-Methoxy-3(3-cyclohexylpropyl)-2,4-dihydroxyquinoline	None
6-Chloro-3(3-cyclohexylpropyl)-2,4-dihydroxyquinoline	None
3(3-Cyclohexylpropyl)-4-hydroxyquinoline	None
3(3-Cyclohexylpropyl)-2,4-dihydroxyquinoline	None
6-Dimethylamino-3(3-cyclohexylpropyl)-2,4-dihydroxyquinoline	None
5,6 (or 6,7-)-Dimethyl-4-hydroxyquinoline	None
6-Aminoquinoline	None
8-Aminoquinoline	None
6-Nitroquinoline	None
8-Nitroquinoline	None
5-Nitroso-8-hydroxyquinoline	None
5-Amino-8-hydroxyquinoline	None
5,7-Dibromo-8-hydroxyquinoline (dissolved in acetone)	3.0
8-Amino-6-methoxyquinoline	None
5-Chloro-7-iodo-8-hydroxyquinoline (dissolved in acetone)	3.0
quinine alkaloid	None

[a] Cleared zone of growth inhibition around solution cup in agar plate, measured in millimeters as radius segment.

possesses an almost specific fungistatic affinity for *Trichophyton mentagrophytes*. Similar findings have been previously described (12), using 8-hydroxyquinoline against a plant fungus and with a different test method. Naturally, the possibility exists that the other investigated quinolines (and quinones) might exhibit fungistatic activities at higher concentrations. The authors' present research is concerned with the mechanism involved in this fungistatic action of 8-hydroxyquinoline, especially as regards the possible inactivation of trace metals necessary for normal fungus growth.

Table IV reveals the wide range of fungistatic activity coefficients of twelve selected compounds. The coefficient of one compound, 2-amino-1,4-naphthoquinonimine hydrochloride, was as much as seventy-seven times higher than that of undecylenic acid, at present a popularly used antifungal agent, with a fungistatic activity coefficient of only 1 (1). Determination of fungistasis, using the two different techniques (Schamberg and Kolmer, Burlingame and Reddish), does not give completely parallel results (1). However, four compounds appeared to be outstanding under the conditions of both test methods, *p*-xyloquinone, *p*-thymoquinone, 8-hydroxyquinoline, and 2-methyl-1,4-naphthoquinone (menadione).

The high fungistatic activity of 2-amino-1,4-naphthoquinonimine hydrochloride, as demonstrated by the Burlingame and Reddish method, could not be duplicated when using the Schamberg and Kolmer technique. The compound is rather unstable and the procedure of heating the agar in which the compound is to be incorporated may facilitate a hydrolysis of the test substance (15). The low value displayed by *p*-toluquinone with this in-

TABLE IV.—FUNGISTATIC ACTIVITY OF TWELVE COMPOUNDS EXPRESSED IN TERMS OF ACTIVITY COEFFICIENTS AND MAXIMUM DILUTION FOR FUNGISTASIS

Compound	Maximum Dilution Method	Fungistatic Activity Coefficients Derived From Dosage-Response Curves (Burlingame and Reddish Method)	
		% Conc. to Give 5-Mm. Clearance	Activity Coefficient
2-Amino-1,4-naphthoquinonimine HCl	1: 35,000	0.013	77.0
p-Xyloquinone	1:100,000	0.020	50.0
p-Thymoquinone	1: 70,000	0.0225	44.4
8-Hydroxyquinoline Benzoate	1: 75,000	0.025	40.0
2-Methyl-1,4-naphthoquinone (Menadione)	1: 60,000	0.0275	36.3
p-Toluquinone	1: 5,000	0.035	28.5
p-Benzoquinone	1: 4,000	0.0775	13.0
1,4-Naphthoquinone	1: 4,000	0.10	10.0
Toluhydroquinone	1: 1,000	0.1275	8.0
2,6-Dichloroquinone	1: 2,500	0.2775	3.6
2,3-Dichloro-1,4-naphthoquinone	1: 35,000	0.2825	3.5
Hydroquinone	1: 2,500	0.5	2.0

of 0.1%. Two of them, 5,7-dibromo-8-hydroxyquinoline and 5-chloro-7-iodo-8-hydroxyquinoline, were dissolved in acetone because of their insolubility in alcohol. The fungistatic activity of the third, 8-hydroxyquinoline, was outstanding, paralleling the most effective fungistatic compounds of the investigated quinone series. All of the substitutions tested, introduction of other hydroxy, methyl, methoxy, amino, nitroso, and nitro groups, and halogens, abolished the activity of the compound. It would seem that 8-hydroxyquinoline

corporation technique was rather surprising in comparison with its relatively high fungistatic activity coefficient with the agar cup-plate method. However, this compound exhibited a lower ceiling value in the dosage-response curves than would appear likely from its activity coefficient. 2,3-Dichloro-1,4-naphthoquinone exhibited considerable fungistasis with this (Schamberg and Kolmer) technique, yet had a low fungistatic activity coefficient. The problem of penetration through the agar medium due to the rather low solubility of this compound

TABLE V.—MAXIMUM NONFUNGICIDAL AND MINIMUM FUNGICIDAL CONCENTRATIONS OF TEN COMPOUNDS, USING A 10-DAY CULTURE OF *Trichophyton Mentagrophytes* ON SABOURAUD'S MALTOSE AGAR PLUS 10% SERUM

Compound	Maximum Nonfungicidal Conc.	Minimum Fungicidal Conc.
2-Amino-1,4-naphthoquinonimine HCl	0.075	0.1
p-Xyloquinone	0.075	0.1
p-Toluquinone	0.1	0.25
2-Methyl-1,4-naphthoquinone	0.1	0.25
p-Thymoquinone	0.1	0.25
1,4-Naphthoquinone	0.1	0.25
p-Quinone	0.5	0.75
8-Hydroxyquinoline Benzoate	0.5	0.75
Toluhydroquinone	5.0	6.0
Hydroquinone	15.0	..

might have been responsible for the discrepancy.

Any laboratory evaluation of an antifungal substance should include specific tests for the determination of its fungicidal potency. The twelve selected compounds were subjected to the authors' modified assay procedure for fungicidal compounds (2). Two compounds, 2,6-dichloroquinone and 2,3-dichloro-1,4-naphthoquinone, could not be evaluated due to their limited solubilities in alcohol. Hydroquinone was not fungicidal even in a 15% concentration and was therefore disregarded as a fungicide. The nine remaining substances showed various grades of fungicidal activity. 2-Amino-1,4-naphthoquinonimine hydrochloride and *p*-xyloquinone, the most active compounds of the group, were fungicidal in a concentration of 0.1%. This is twenty-five times higher than undecylenic acid, which was found to be fungicidal in a 2.5% concentration under the same test conditions. 8-Hydroxyquinoline, which exhibited excellent fungistatic properties, was fungicidal in a concentration of 0.75%.

The fungicidal activities indicated here are valid only when employing 95% alcohol as the solvent. Any decrease in the alcohol concentration necessitates an increase in the concentration of the test material, providing all of the other test conditions are identical.

SUMMARY

1. Eleven derivatives of benzoquinone, twenty derivatives of naphthoquinone, and twenty-four quinoline derivatives, dissolved in 95 per cent ethyl alcohol in a concentration of 0.1 per cent, were evaluated for their fungistatic activities.

2. Twelve selected compounds were subjected to more intensive laboratory investigation to determine their fungistatic activity coefficients and their fungicidal properties.

3. It could be shown that four compounds of this series, *p*-xyloquinone, *p*-thymoquinone, 2-methyl-1,4-naphthoquinone (menadione), and 8-hydroxyquinoline, possess outstanding antifungal qualities.

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CORRECTION

In the article entitled "Digitalis VII. The Effect of Various Alkalies on the Sensitivity of the Baljet Reaction for Digitoxin", THIS JOURNAL, 37, 297–301(1948) Table I, page 299, should read as follows:

TABLE I.—OPTICAL DENSITY × 100

Expt.	NaOH Conc., %	Picric Acid Conc., %	Time (in Minutes) After Addition of Reagent 5	10	20	30	40	50	60	70	80	90	120	150	180
1	0.25	0.5	25.0	30.5	35.0	36.5	37.4	37.5	37.1	37.0	37.1	37.1	37.0	36.6	36.5
2	0.5	0.5	37.5	39.5	39.0	38.9	39.0	38.9	38.4	38.7	38.0	38.0	38.0	37.5	37.1
3	0.5	0.25	35.7	39.6	40.1	40.1	40.2	40.0	39.4	39.4	39.5	39.0	38.3	37.6	36.5

Enhancement of Penicillin Activity *in vitro* by Vitamin K_5*,†

By ROBERTSON PRATT, JEAN DUFRENOY, and PETER P. T. SAH
with the laboratory assistance of VIRGINIA L. PICKERING

Vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride) in concentrations from 0.1 to 10 mg./L. enhances the effectiveness of penicillin *in vitro* against *Staphylococcus aureus, Proteus vulgaris,* and *Escherichia coli.* Depending upon the test organism used, the zones of inhibition around cylinders containing a solution with 1 unit penicillin and 1 microgram K_5/ml. were as large as could be obtained around cylinders containing penicillin alone in concentrations ranging from 1.5 to 100 units/ml. Since the activity of combinations of vitamin K_5 and of penicillin in suitable concentrations is greater than would be expected from the sum of the activities of the two compounds acting separately, the effect appears to be one of synergism rather than merely the result of two antibacterial agents acting simultaneously but independently.

An agent that is capable of increasing the effectiveness of penicillin is potentially a valuable adjunct to the clinicians *armamentarium.* Reports of the enhancement of the antibiotic activity of penicillin by various agents have been in the literature since 1944 when Cornman (1, 2) and Lewis (3) independently observed that partially purified penicillin exerted a selective lethal action *in vitro* against rat and mouse sarcoma cells whereas pure penicillin possessed no such activity. In 1945 Dunham and Rake (4) showed that partially purified penicillin reduced the motility of cells of *Treponema pallidum* and that crystalline penicillin did not do so. Independently, Smith (5) and de Ropp (6) reported retardation of seed germination and of root growth in solutions of crude or of partially purified penicillin. Among the impurities that have been isolated from amorphous penicillin and that might be expected to induce the observed results are indole acetic acid, phenylacetic acid, orthohydroxyphenylacetic acid, members of vitamin complexes, and other unidentified growth-regulating factors. These are discussed in a recent review (7). Recently Lecoq and Solomides (8) reported that only one-half as much penicillin or streptomycin was required to inhibit growth of *Escherichia coli* in nutrient broth containing one part of para-amino-benzoic acid in 150 parts of broth as in broth lacking PAB.

Turning to *in vivo* studies, Welch, Randall, and Price (9) reported the occurrence in a number of commercial lots of amorphous penicillin of an unidentified factor which, though devoid of protective activity itself, enhanced the protective action of penicillin against *Eberthella typhosa* in mice as much as 100 per cent in some cases, i.e., 2000 units of amorphous penicillin containing the factor or of crystalline penicillin with the factor added were as effective as 4000 units of crystalline penicillin alone. Pursuing that line of investigation, Hobby, Lenert, and Hyman (10) observed that impure penicillin was 3 to 5 times more effective than crystalline penicillin G in protecting mice against *Streptococcus hemolyticus.* Likewise, impure penicillin afforded greater protection against *Diplococcus pneumoniae* infections in mice. The factor reported by Welch, *et al.*, and Hobby, *et al.*, appeared not to be correlated with the amount of phenylacetic acid present or with degradation products of penicillin, although other observations have indicated that phenylacetic acid, sodium acetate, and perhaps even normal physiological saline may enhance the activity of crystalline benzyl penicillin under certain conditions (11). Hobby, *et al.* also observed that the chemotherapeutic activity of crystalline penicillin G may be enhanced by a number of dissimilar substances, among which are penicillin K and inactivated penicillinase.

It has been shown in this laboratory that appropriate trace amounts of cobalt, nonbacteriostatic *per se,* increase at least twofold the protective action of crystalline penicillin G against infection of mice by *E. typhosa* (12). The enhancement of penicillin action by cobalt differs from the enhancement induced by the factor reported by Welch, *et al.* (9), Hobby, *et al.* (10), and others (11) in that it is readily demonstrated *in vitro* (13) and in that the concentrations of cobalt required to produce the effect are extremely low.

The trend in the pharmaceutical industry and in medicine has ever been away from the use of unidentified or crude preparations and toward the goal of administration of definite amounts of known compounds. Thus, if the use of adjuvants to enhance or to potentiate the action of penicillin should some day be deemed clinically

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† This research was supported in part by a generous grant from the Cutter Laboratories, Berkeley, Calif

practicable, it would seem desirable to offer the profession chemically defined compounds. The observations on cobalt (12, 13) represented such an advance. However, although the concentrations of cobalt that were used were well below the toxic level, some opposition to its use in clinical practice might be anticipated because of its potential toxicity in the event of misuse. Therefore, the discovery of other agents that are capable of enhancing penicillin activity but that are potentially less dangerous would be most welcome. The present paper reports the use of such a compound, i.e., vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride), which is well known for its antihemorrhagic properties. Veldstra and Wiardi (14) reported the *LD* for oral administration in rats as being 0.7 Gm./Kg. However, they failed to state whether this figure represented the LD_{50} or LD_{100}. Sah and Anderson (unpublished data) found the LD_{50} for mice to be 750 mg./Kg. for intraperitoneal administration.

EXPERIMENTAL

Vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride) was synthesized as previously described by one of us (15) with slight modifications to improve the yield.

Estimates of the antibacterial effectiveness of the penicillin, a preparation of crystalline sodium benzyl penicillin that assayed 1600 units/mg., were made by the cylinder-plate technique, using both the standard overnight assay and the rapid assay involving physical development (16). *Staphylococcus aureus* NRRL 313, *Proteus vulgaris* HX19, and a laboratory isolate identified as *Escherichia coli* were used as test organisms.

RESULTS

Vitamin K_5 Incorporated in Test Agar.—Incorporation of appropriate amounts of vitamin K_5 in the nutrient agar resulted in formation of larger zones of inhibition than occur normally around cylinders containing a given concentration of penicillin on assay plates seeded with *S. aureus*, with *P. vulgaris*, or with *E. coli*. The apparent enhancement of penicillin activity occurred whether the assay was performed by means of the standard procedure or by means of the rapid cylinder-plate technique described previously (16). The results are summarized in Figs. 1 to 3.

The enhancement was generally greater at the lower concentrations of penicillin, and was more pronounced on plates seeded with Gram-negative *E. coli* or *P. vulgaris* than on those seeded with *S. aureus*. For example, the maximum enhancement of activity against *S. aureus* was less than two-fold but a ten- to twenty-fold increase in activity was noted against *E. coli*: on five-hour assay plates with 1 mg. K_5/L. (1 p. p. m.) a solution with 0.5 unit penicillin/ml. produced zones equivalent to those produced by 5 units/ml. on the regular me-

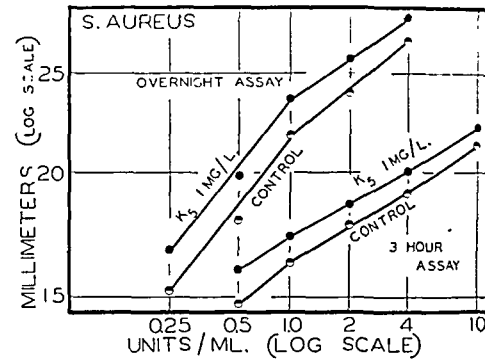

Fig. 1.—Diameter of inhibition zones formed around cylinders with different concentrations of penicillin tested against *Staphylococcus aureus* seeded on the standard medium and on the same medium containing vitamin K_5.

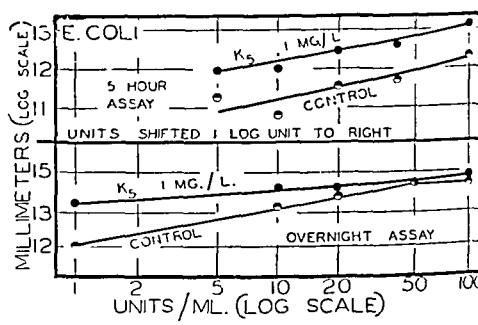

Fig. 2.—Diameter of inhibition zones formed around cylinders with different concentrations of penicillin tested against *Escherichia coli* seeded on the standard medium and on the same medium containing vitamin K_5.

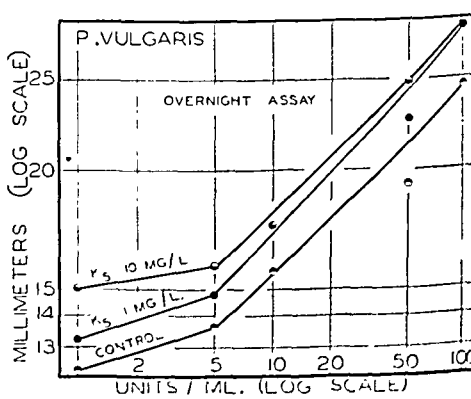

Fig. 3.—Diameter of inhibition zones formed around cylinders with different concentrations of penicillin tested against *Proteus vulgaris* seeded on the standard medium and on the same medium containing vitamin K_5.

dium, and in overnight assays 1 unit penicillin/ml. produced zones on K_5 medium equivalent to zones produced by 20 units penicillin/ml. on standard medium. The maximum increase in penicillin effectiveness against *P. vulgaris* was approximately eightfold.

Vitamin K_5 Dissolved in Solutions of Penicillin.—Vitamin K_5 and some of its analogs and related compounds are known to inhibit the growth and other metabolic activities of several species of bacteria, fungi, yeasts, and algae (17–22). Therefore, it seemed of interest to ascertain whether the effect noted above was actually due to enhancement of penicillin activity or whether it should be attributed merely to the additive effect of two antibacterial agents acting simultaneously.

Accordingly, solutions with different concentrations of vitamin K_5 and of penicillin were assayed by the standard cylinder-plate technique (sixteen hours' incubation and standard medium) using *S. aureus* and *E. coli* as the test organisms. The averaged results of this series of experiments plotted in Fig. 4 suggest that in appropriate concentrations vitamin K_5 and penicillin act synergistically, since the combined antibacterial action of the two agents was greater than would be expected from the sum of their independent actions. This was most pronounced in the case of the penicillin-resistant, Gram-negative *E. coli*. A solution containing 1 mg. K_5/L. (1 p. p. m.) produced zones of inhibition approximating in diameter the zones produced by a solution containing 0.5 unit penicillin/ml. But a preparation with 0.5 unit penicillin/ml. fortified with 1 mg. K_5/L. yielded inhibition zones larger than those produced by 10 units penicillin/ml. alone, and the zones produced around cylinders with 1 unit penicillin/ml. similarly fortified with K_5 were larger than those produced by 100 units penicillin/ml.

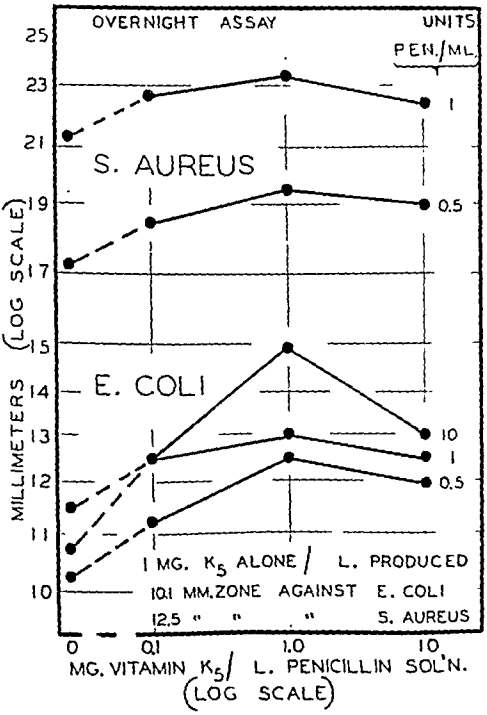

Fig. 4.—Diameters of inhibition zones formed around cylinders with different concentrations of penicillin and of vitamin K_5 tested against *Staphylococcus aureus* and against *Escherichia coli* seeded on the standard medium.

In the case of plates seeded with *S. aureus* the effect was not so pronounced. Vitamin K_5 alone in the penicylinders at a concentration of 1 mg./L. (1 p. p. m.) produced zones that averaged 12.5 ± 0.4 mm. in diameter. Such zones are considerably smaller than those produced by a penicillin solution containing 0.25 unit/ml. Addition of 1 mg. K_5/L. to solutions containing 0.5 or 1 unit penicillin/ml. gave zones equal to those produced by 0.75 and 1.4 units penicillin/ml., respectively.

The enhancement of penicillin activity by vitamin K_5 was noted with concentrations of the latter ranging from 0.1 mg. to 10 mg./L. but a concentration of 1 mg./L. gave maximum enhancement against *E. coli* and *S. aureus*. The optimum concentration of K_5 for increasing the effectiveness of penicillin against *P. vulgaris* was not determined but it appears to be greater than 1 p. p. m. (see Fig. 3).

SUMMARY

A study was made of the effect of vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride) in enhancing the activity of benzyl penicillin against *Staphylococcus aureus*, *Proteus vulgaris*, and *Escherichia coli*. Tests were performed by the cylinder-plate technique.

The magnitude of the enhancement depended upon the conditions of the experiment, the concentrations of K_5 and of penicillin, and the test organism. Under optimal conditions K_5 incorporated in the nutrient agar (1 mg. K_5/L.) increased the apparent activity of penicillin approximately twofold against *S. aureus*, eightfold against *P. vulgaris*, and twenty-fold against *E. coli*. Solutions containing 1 unit of penicillin and 1 microgram of K_5/ml. when placed in penicylinders on standard agar exerted activity against *S. aureus* equivalent approximately to the effect of 1.4 units penicillin/ml. alone. When *E. coli* was the test organism the same solution produced an effect greater than that of 100 units penicillin/ml.

The optimum concentration of K_5 for enhancing the effectiveness of benzyl penicillin is about 1 microgram/ml. of penicillin solution for *S. aureus* and *E. coli*, although the enhancing effect obtains over the range 0.1 to 10 micrograms/ml.

The activity of combinations of vitamin K_5 and of penicillin in suitable concentrations is greater than would be expected from the sum of the activities of the two agents acting separately. Therefore, it is concluded that vitamin K_5 and penicillin act synergistically.

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Book Reviews

Toxicology and Hygiene of Industrial Solvents. Edited by K. B. LEHMAN and F. FLURY. Translated from the German by ELEANOR KING and HENRY F. SMYTH, JR. The Williams & Wilkins Company, Baltimore, 1943. xiv + 378 pp. 14.5 x 23 cm. Price $5.

Perhaps the most common chemical industrial hazard arises from the misuse of industrial solvents. The authors have brought together the factors of interest in industrial toxicology in this field.

The first portion of this book is concerned with the chemistry (including analysis), the preparation, and the use of solvents. The general toxicology of solvents is then considered. This is followed by the main section of the book which deals with the individual solvents. Hydrocarbons, chlorinated hydrocarbons, alcohols, esters, ethers, aldehydes, ketones, terpenes, and miscellaneous solvents are considered here. A short chapter on skin injuries follows and this in turn is followed by sections on preventive measures and official German regulations.

At first glance this book appears to be of limited pharmaceutical interest. Further study shows considerable data on glycols, ethyl acetate, dibutylphthallate, dimethylphthallate, ether, chloroform, carbon tetrachloride, and many other compounds used in pharmacy. In addition the discussions of experimental toxicology may serve as points of departure for other studies by the research worker.

Chromatography. By H. G. CASSIDY, N. APPLEZWEIG, S. CLAESSON, V. R. DEITZ, B. J. MAIN, A. J. P. MOORE, R. L. PECK, W. A. SCHROEDER, L. SHEDLOVSKY, W. H. STEIN, H. C. THOMAS, and L. ZECHMEISTER. Annals of the New York Academy of Sciences, February 10, 1948. 186 pp. 15 x 23.5 cm.

This booklet represents the report of a conference on chromatography held at the New York Academy of Sciences.

Dr. Zechmeister, one of the early founders of th interesting method of analysis, wrote the introductory chapter on the history, scope, and basic methods. Frontal analysis and experiments in systematic quantitative methods follow. After a section on the applications of chromatography to the problem of spacial configurations and to streptomycin, methods of partition chromatography are discussed including their specific application to amino acid analysis. Ion exchange adsorbents and classifications of solid adsorbents are included.

The review of recent developments in this field forms a useful supplement to reference books and texts in chromatography.

Blood Derivatives and Substitutes. By CHARLES STANLEY WHITE and JACOB JOSEPH WEINSTEIN. The Williams & Wilkins Company, Baltimore, Md., 1947. xviii + 484 pp. 15 x 22.5 cm. Price $7.50.

The dramatic role of blood and blood plasma in World War II is fresh in the mind of everyone. Th availability of human blood in large quantities through the blood bank programs led to many developments in fractionation. The research on these fractions, which is explained in this book, led to the development of many unique uses.

This book covers, in addition to a historical co and a brief section on the chemistry and physiol of plasma, chapters on the preparation of liq citrated plasma, storage, drying of plasma, adm. istration, and fractionation. A chapter dealing with plasma substitutes covers such substances as gelatin pectin, and acacia. There are also chapter shock, clinical results with human plasma, an toward reactions with plasma.

The addition of about 200 graphs, charts, pictures aids in making this an authoritative in this important field.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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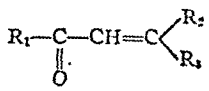
The Antibacterial Activity of Some Beta-Aroyl Acrylic Acids, Esters and Amides*

By BETTY JANE CRAMER[1], WILLIAM SCHROEDER[2], WILLIAM J. MORAN[3], CYRIL H. NIELD[4], MARGUERITE EDWARDS[5], CHARLES I. JAROWSKI[6], and BRUNO PUETZER†

Methods of preparing a series of ring-substituted beta-aroyl acrylic acids, esters, and amides are described. The bacteriological properties of these compounds are reported. Compounds containing alkyl groups in the ortho positions relative to the unsaturated side chain were found to be most effective.

IN 1945 Geiger and Conn (1) postulated a mechanism for the antibiotic action of clavacin and penicillic acid, which is dependent upon their reaction with the sulfhydryl groups in bacterial enzyme systems or with sulfhydryl-containing metabolites essential to bacteria. This theory originated from their observation that both of these compounds contain a common structural feature; namely, the —CH=C—C=O group. In support of their claims they demonstrated that clavacin and penicillic acid are inactivated by an excess of a sulfhydryl compound and further that each of these compounds, when present in excess, abolishes the nitroprusside reaction of cysteine or thioglycollate. A similar inactivation has also been reported by other investigators (2, 3).

Based on chemical studies on the addition reactions of α,β-unsaturated ketones, Geiger and Conn (1) concluded that a ketone will undergo addition reactions most readily when R_1 of Formula I is an aromatic radical and R_2 and R_3 are hydrogens.



(I)

In conformance with the above postulate they found that acrylophenone (R_1 = phenyl; R_2 and R_3 = H) was actively bacteriostatic and that it imitated clavacin since its activity was eliminated by the presence of cysteine or thioglycollate.

In the course of an investigation to prepare benzenoid homologs of clavacin it was found that treatment of 2-carbethoxy-γ-chromanone (II) with sodium hydroxide gave β-(*o*-hydroxyben-

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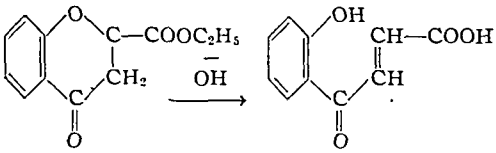
† The authors wish to thank Dr. M. K. McPhail for the toxicity data. For the analytical values we are indebted to Mr. Glenn B. Hess and Miss Celia Durham. To Dr. Thomas C. Grubb we express our gratitude for his many suggestions and consistent assistance.

zoyl)-acrylic acid (III). This reaction is analogous to the conversion of the flavanone, hesperitin, to 2,4,6-trimethoxyphenyl-3,4-dimethoxystyryl ketone by means of dimethyl sulfate and potassium hydroxide (4).



(II) (III)

A mechanism for the opening of the pyran ring has been suggested in a recent paper (5).

In compound (III) can be seen the structure which Geiger and Conn (1) suggested for effective antibacterial action. From Table I it is evident that β-(o-hydroxybenzoyl)-acrylic acid exhibited a marked bacteriostatic activity against both Gram-positive and Gram-negative organisms.

Since the β-benzoyl acrylic acids as a class would represent an improvement over the acrylophenone structure in so far as stability, solubility, and ease of preparation are concerned, a series of β-aroyl acrylic acids and related compounds was prepared in an effort to find compounds with even greater antibacterial activity. These compounds are listed in Tables II, III, IV, and V.

ene, tetrachlorethane or an excess of the benzenoid compound). Aluminum chloride (anhydrous) was used in 0.2 mole excess after molar quantities had been calculated to react with the oxygen of phenols, ethers, and carbonyl groups. During the slow addition of aluminum chloride, the mixture was kept at various temperatures ranging from 10° to 25° depending on the vigor of the reaction. Mechanical stirring was continued at room temperature or up to 100° from one to three hours. When the evolution of HCl subsided the reaction mixture was decomposed with dilute HCl. In some cases the acid precipitated from the solvent or from the aqueous acid solution after the removal of the organic solvent by steam distillation. The product was purified by reprecipitation from a charcoaled sodium bicarbonate solution and recrystallization from benzene, alcohol, dilute alcohol, or glacial acetic acid.

Procedure II: Preparation of Esters.—The esters were prepared from the acids by refluxing with alcoholic compounds containing 2% anhydrous HCl as a catalyst.

Procedure III: Preparation of Amides and Anilides.—The β-aroyl acrylic acid chloride, prepared from the acid (1 mole) and phosphorous pentachloride (1 mole), was treated at 0–15° with the amine (1 mole) and pyridine (2 moles). The reaction was carried out in anhydrous ether or dioxane depending upon the solubility of the amine. When ether was used as a solvent, the reaction mixture was extracted with dilute hydrochloric acid and saturated sodium bicarbonate solution, dried, and then the ether removed. The residue was treated with a mixture of "Skellysolve A" and ether; the product was filtered off and recrystallized from either dilute acetic acid, benzene, or nitromethane.

When dioxane was used as a solvent, the reaction mixture was drowned in dilute hydrochloric acid and the precipitated solid filtered off. The solid was ground in a mortar with a saturated sodium bicarbonate solution, filtered, washed with water, dried, and recrystallized.

Procedure IV: Addition of Active Hydrogen Compounds.—These reactions were carried out by

TABLE I.—THE BACTERIOSTATIC ACTION OF β-(o-HYDROXYBENZOYL)-ACRYLIC ACID

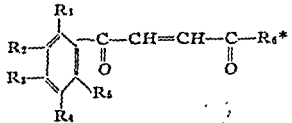
Organism	Serial Dilution Test Method[d]					
	1 × 10⁴	2 × 10⁴	4 × 10⁴	8 × 10⁴	16 × 10⁴	32 × 10⁴
Staphylococcus aureus	−[a]	−	−	−	+[b]	+
Staphylococcus albus	−	−	−	−	+	+
Bacillus subtilis	−	−	−	−	+	+
Escherichia coli	+[c]	+	+	+	+	+
Eberthella typhosa (Hopkins)	−	−	−	−	+	+
Bacillus prodigiosum	±	±	±	+	+	+
Salmonella paratyphi A	−	−	−	+	+	+
Salmonella paratyphi B	−	−	−	±	+	+
Shigella paradysenteriae (Flexner)	−	−	−	+	+	+
Shigella sonne	...	+	+	±	+	+
Trichophyton mentagrophytes (*spore suspension*)	+	+	+	+	...	...

[a] Complete inhibition.
[b] No inhibition.
[c] Partial inhibition.
[d] Method of testing (Serial Dilution Test).—*Medium:* Neopeptone-dextrose broth for fungi, F D A nutrient broth for bacteria. *Procedure:* Make 1: 1,000,000 dilution of culture in broth. Pipette 1 ml. inoculated broth into sterile 10 × 70-mm. tubes. To first tube add 1 ml. solution to be tested, mix well with pipette and remove 1 ml. to second tube. Repeat until the required number of dilutions is made. Leave last tube fc [illegible] d after incubating for exactly twenty-four hours at 37°. Incubate fungi cultures for ten day [illegible] soluble in water were submitted for testing in dilute alcohol. Only those dilutions which cor [illegible] of alcohol were considered, for higher concentrations were found to be inhibitory to the growth of *S. aureus* and *E. typhosa*.

EXPERIMENTAL

Preparation of Compounds

TABLE II.—THE BACTERIOSTATIC ACTION OF SOME β-BENZOYL ACRYLIC ACIDS, ESTERS, AND AMIDES



No.	R_1	R_2	R_3	R_4	R_5	R_6	M. p. (Unc.), ° C.	B. p. (Unc.), ° C.	Method of Prep.	Highest Dilution Causing Inhibition of Growth† *S. aureus***	*E. typhosa***
1	H	H	H	H	H	OH	96[a]	.....	I	1:40,000	.....[b]
2	H	H	Me	H	H	OH	136– 7[c]	.....	I	1:40,000	.....
3	Me	H	Me	H	Me	OH	141[d]	.....	I	1:200,000	.....
4	Et	H	Et	H	Et	OH[e]	100– 1.5	.....	I	1:400,000	1:100,000
5	H	H	Cl	H	H	OH[f]	159–61	.....	I	1:40,000	1:80,000
6	Cl	H	Cl	H	H	OH[g]	192– 4	.....	I	1:40,000	1:20,000
7	H	H	Cl	Cl	H	OH[h]	143	.....	I	1:80,000	1:20,000
8	OH	H	H	H	H	OH[i]	184	.....	..	1:80,000	1:80,000
9	H	H	OMe	H	H	OH	134[j]	.....	I	1:40,000	.....
10	H	H	HCONH	H	H	OH[k]	244– 5	.....	I	.....	.....
11	H	H	CH_3CONH	H	H	OH[l]	239	.....	I	.....	.....
12	H	NO_2	H	H	H	OH[m]	201– 2	.....	..	.....	.....
13	OH	H	H	Cl	H	OH	191	.....	I	1:40,000	1:40,000
14	OH	Br	H	Br	H	OH[n]	193– 5	.....	I	.....	.....
15	Me	H	H	CH_3CONH	H	OH[o]	184	.....	I	.....	.....
16	Me	CH_3CONH	H	H	H	OH	210–13	.....	I	.....	.....
17	OH	CH_3CONH	H	H	H	OH[p]	205– 6	.....	I	.....	.....
18	OH	H	H	CH_3CONH	H	OH	197– 8	.....	I	.....	.....
19	Me	H	OH	$(CH_3)_2CH$	H	OH	160	.....	I	.....	.....
20	CH_3COO	H	H	Me	H	OH	134	.....	I	1:80,000	1:20,000

* The open structure has been assigned to all these compounds on the basis of a polarographic study carried out on compounds 1, 3, 26, 30, 35, and 42 (8). These data showed half-wave potential values indicating reduction of the ketone group, thus eliminating the possibility that at least these six compounds did not exist in the cyclic form. This is in conformance with the findings of others (9).

† For the method of testing see Table I.

** *Staphylococcus aureus* No. 209 and *Eberthella typhosa* (Hopkins), 24-hr. cultures.

a M. p. reported, 96–7° (10). The activity has been reported by H. Rinderknecht, *et al.*, as 1:25,000 (11).

b No activity in lowest dilution tested.

c M. p. reported, 138° (12).

d M. p. reported, 140.5° (13).

e Calcd. for $C_{18}H_{20}O_3$: N. E., 260. Found: N. E., 257.

f M. p. reported, 157–8°. The activity has been reported as 1:50,000 by Rinderknecht, *et al.* (11).

g M. p. reported, 191–3°. The activity reported was 1:80,000 by Rinderknecht, *et al.* (11).

h Calcd. for $C_{10}H_6O_3Cl_2$: C, 49.01; H, 2.47. Found: C, 49.36; H, 2.70. Oxidation with alkaline permanganate gave 3,4-dichlorbenzoic acid, m. p. 202–3°; m. p. reported, 201–2° (14).

i Calcd. for $C_{10}H_8O_4$: C, 62.5; H, 4.19. Found: C, 62.12; H, 4.35.

j M. p. reported, 134° (15).

k Calcd. for $C_{11}H_9O_4N$: C, 60.27; H, 4.14. Found: C, 60.24; H, 4.58.

l Calcd. for $C_{12}H_{11}O_4N$: C, 61.7; H, 4.8; N, 6.01; N. E., 233. Found: C, 61.73; H, 4.94; N, 6.02; N. E., 234. Oxidation with alkaline permanganate gave *p*-acetylamino benzoic acid, m. p. 251°; m. p. reported, 250° (16).

m Prepared by nitration of β-benzoyl acrylic acid. Oxidation with alkaline permanganate gave *m*-nitrobenzoic acid, m. p. 141°; m. p. reported, 140–1° (17).

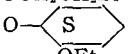
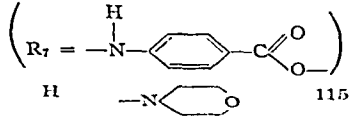
n Calcd. for $C_{10}H_6O_4Br_2$: C, 34.3; H, 1.75. Found: C, 34.2; H, 1.75.

o Calcd. for $C_{12}H_{11}O_4N$: C, 63.16; H, 5.26; N. E., 247. Found: C, 63.34; H, 5.68; N. E., 247.

p Calcd. for $C_{12}H_{11}O_3N$: N, 5.63; N. E., 249. Found: N, 5.55; N. E., 123. The phenolic group was titrated also.

(*Cont'd on p. 442*)

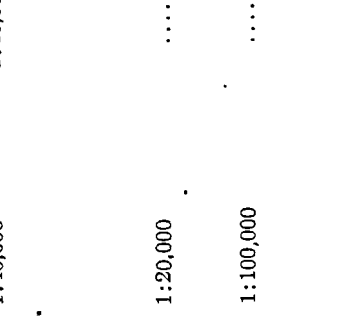
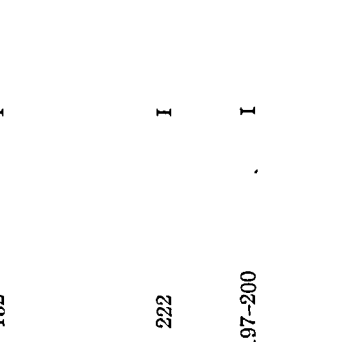
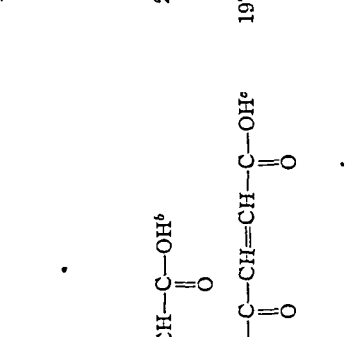
TABLE II.—THE BACTERIOSTATIC ACTION OF SOME β-BENZOYL ACRYLIC ACIDS, ESTERS, AND AMIDES—(*Cont'd from p. 441*)

No.	R_1	R_2	R_3	R_4	R_5	R_6	M. p. (Unc.), °C.	B. p. (Unc.) °C.	Method of Prep.	Highest Dilution Causing Inhibition of Growth† *S. aureus***	*E. typhosa***
21	OH	Me	H	Me	H	OH[q]	195–96	.....	I	.....	.....
22	OH	H	Me	H	H	OH	170– 2	.....	I	1:80,000	.....
23	H	Me	OH	H	H	OH	168–70	.....	I	1:20,000	.....
24	OH	Me	H	H	H	OH[r]	176– 7	.....	I	1:300,000	1:20,000
25	OH	H	H	Me	H	OH[s]	174– 6	.....	I	1:80,000	1:20,000
26	H	Me	Cl	H	H	OH[t]	126– 8	.....	I	1:160,000	1:40,000
27	OH	H	Me	Cl	H	OH[u]	206– 9	.....	I	1:160,000	1:40,000
						Esters					
28	H	H	H	H	H	OMe	....	138–40 (3)[v]	II	1:200,000	.....
29	H	H	H	H	H	OEt	54[w]	.....	..	1:400,000	1:200,000
30	H	H	H	H	H	OEt	....	120–25 (2)[x]	II	1:400,000	1:200,000
31	H	H	H	H	H	$OCH_2CH{=}CH_2$	....	146– 8 (2)	II	1:400,000	.....
32	H	H	H	H	H	OCH_2CH_2Cl[y]	....	163– 5 (3)	II	1:800,000	.....
33	H	H	H	H	H	OCH_2CH_2Br[z]	54	168–70 (1)	II	1:400,000	.....
34	H	H	H	H	H		....	178–80 (1)	II	1:800,000	1:100,000
35	Me	H	Me	H	Me	OEt	....	155– 6 (3)	II	1:400,000	.....
36	Me	H	Me	H	Me	OCH_2CH_2Cl	....	168–70 (1.5)	II	1:800,000	.....
37	Me	H	Me	H	Me	OBz	....	225 (0.8)	II	.....	.....
38	Me	H	Me	H	Me	$O{-}CH_2{-}CH_2{-}N(Et){-}Et \cdot HCl$	84–90	.....	II	.....	.....
39	Cl	H	H	H	H	OEt[aa]	59–60	156– 8 (0.7)	II	1:400,000	1:200,000
40	Cl	H	Cl	H	H	OEt	....	146 (0.5)	II	1:800,000	1:100,000
41	H	H	CH_3CONH	H	H	OEt	118–21	.....	II	1:400,000	1:100,000
42	H	Me	Cl	H	H	OEt	....	145–50 (0.3)	II	1:600,000	1:200,000
43	H	Me	Cl	H	H	$O{-}CH_2{-}CH_2{-}N(Et){-}Et \cdot HCl$	143–5	.....	II	.....	.....
44	OH	Me	H	H	H	OMe	118–20	.....	II	.....	1:100,000
45	OH	Me	H	H	H	OEt	82– 5	.....	II	.....	1:100,000
46	OH	H	H	Me	H	OMe	100	.....	II	.....	.....
47	OH	H	H	Me	H	OEt	60	.....	II	1:100,000	1:100,000
48	OH	H	Me	H	H	OMe	110–14	.....	II	.....	1:100,000
						Amides					
49	H	H	H	H	H		115	.....	III	1:200,000	1:200,000

						$N(CH_3)_2$					
51	Me	H	Me	H	Me	NH_2[cc]	153–4	.....	III	1:400,000	.....
52	Me	H	Me	H	Me	—NH[dd]	201–2	.....	III	1:800,000	.....
53	Me	H	Me	H	Me	R_7H[ee]	257–9	.....	III	1:800,000	.....
54	Me	H	Me	H	Me	R_7Et[ff]	154–5	.....	III	1:2,000,000	.....[jj]
55	Me	H	Me	H	Me	R_7—$(CH_2)_2$[gg]	142–5	.....	III	1:200,000	.....
						Et—N—Et					
						HCl					
56	Me	H	Me	H	Me	$R_7(CH_2)_2CH_3$[hh]	113–6	.....	III	1:800,000	.....
57	Me	H	Me	H	Me	$R_7(CH_2)_3CH_3$	127–8	.....	III	1:800,000	.....
58	Me	H	Me	H	Me	NH[ii]	258–9	.....	III	1:1,600,000	.....
						SO_2NH_2					

q Calcd. for $C_{19}H_{18}O_4$: C, 65.45; H, 5.45. Found: C, 65.23; H, 5.66.
r Calcd. for $C_{11}H_{10}O_4$: C, 61.06; H, 4.88; N. E., 206. Found: C, 63.92; H, 5.10; N. E., 210.
s Calcd. for $C_{11}H_{10}O_4$: C, 61.06; H, 4.88. Found: C, 63.88; H, 4.88.
t Calcd. for $C_{11}H_9O_3Cl$: C, 58.8; H, 4.03. Found: C, 58.82; H, 4.11. Oxidation with alkaline permanganate gave 4-chloro-isophthalic acid, m. p. 294°; m. p. reported, 294.5° (18).
u Calcd. for $C_{11}H_9O_4Cl$: N. E., 240. Found: N. E., 245.
v B. p. reported, 186° (16 mm.) (19).
w M. p. reported, 51° (20).
x B. p. reported, 181–5° (25 mm.) (20).
y Calcd. for $C_{12}H_{11}O_3Cl$: Cl, 14.7. Found: Cl, 14.82.
z Calcd. for $C_{12}H_{11}O_3Br$: C, 50.88; H, 3.89. Found: C, 50.67; H, 4.2.
aa Calcd. for $C_{13}H_{11}O_3Cl$: Cl, 14.88. Found: Cl, 14.48.
bb Calcd. for $C_{18}H_{18}O_2N_2$: C, 73.41; H, 6.16. Found: C, 73.43; H, 6.6.
cc Calcd. for $C_{13}H_{19}O_2N$: C, 71.86; H, 6.95. Found: C, 72.07; H, 7.33.
dd Calcd. for $C_{18}H_{19}O_2N$: N, 4.77. Found: N, 4.73.
ee Calcd. for $C_{22}H_{19}O_4N$: C, 71.2; H, 5.6. Found: C, 70.88; H, 5.14.
ff Calcd. for $C_{22}H_{21}O_4N$: C, 72.3; H, 6.34. Found: C, 72.06; H, 6.03.
gg Calcd. for $C_{24}H_{28}O_4N_2Cl.HOH$: C, 65.4; H, 7.2. Found: C, 65.52; H, 7.11.
hh Calcd. for $C_{22}H_{23}O_4N$: C, 72.79; H, 6.64. Found: C, 72.71; H, 6.84.
ii Calcd. for $C_{18}H_{20}O_4N_2S$: C, 60.83; H, 5.5. Found: C, 61.26; H, 5.4.
jj Raistrick Test (modified) for testing compounds which show marked activity in the screen test was used as follows:
Media.—F. D. A. Nutrient broth.
Organism.—*S. aureus* 209 and *E. typhi* (Hopkins, also others as requested).
Procedure.—Pipette 4 ml. broth into sterile 125 × 25-mm. tubes. Make serial dilutions of the solution to be tested in sterile 22 × 150-mm. test tubes with sterile distilled water. To each tube of broth add 1 ml. of the dilution. *Example:* First dilution 1:1000, after added to broth 1:5000. Second dilution 1:2000, after added to broth 1:10,000, etc.
Control.—1 ml. sterile distilled water added to 4 ml. broth.
Culture.—Dilute each culture (if good growth) 1:1000. With 0.2-ml. pipette add 0.01 ml. of diluted culture to each tube and controls. Incubate twenty-four hrs. at 37°.

TABLE III.—THE BACTERIOSTATIC ACTION OF SOME β-AROYL ACRYLIC ACIDS

No.	Compound	M. p. (Unc.), ° C.	Method of Prep.	Highest Dilution Causing Inhibition of Growth *S. aureus*	*E. typhosa*
59		152	I	1:40,000	1:40,000
60		222	I	1:20,000	....
61		197–200	I	1:100,000	....

[a] Calcd. for $C_{11}H_{10}O_5S$: C, 52.7; H, 3.32; N. E., 182. Found: C, 52.62; H, 3.40; N. E. 180.
[b] Calcd. for $C_{20}H_{12}O_7$: N. E., 182. Found: N. E., 201.
[c] Calcd. for $C_{28}H_{19}O_6N$: N. E., 181. Found: N. E., 178.

mixing the β-aroyl acrylic acids or esters with equimolar quantity of the HA compound (HA compound containing active hydrogen) in alcoh In some instances it was advantageous to react t sodium salts of the acids with the HA constitu in water. The adducts usually crystallized out standing at room temperature. Where salts w used, acidification caused the separation of t addition compound. The products were purifi by recrystallization from ethanol, dilute ethanol, benzene.

Preparation of 2-Carbethoxy-γ-Chromanone. One-tenth mole (21.8 Gm.) of 2-carbethoxy-chromanone (6) was dissolved in 100 ml. of 95 ethanol and hydrogenated in the presence of o teaspoonful of Raney nickel. The pressure dropp from 50 to 40.3 lb. in twenty minutes (theoreti drop = 9.5 lb.). The catalyst was filtered off a the 2-carbethoxy-γ-chromanone was purified by d tillation. Nineteen grams (86% yield) of a liqu was obtained; b. p. 3 = 153–4°.

Anal.—Calcd. for $C_{12}H_{12}O_4$: C, 65.5; H, 5.4 Found: C, 65.43; H, 5.58 (7).

Preparation of β-(o-Hydroxybenzoyl)-Acryl Acid.—The above chromanone ester (19 Gm.) w dissolved in 40 ml. of 95% ethanol, and treated wi 45 ml. of 20% sodium hydroxide at 50° for one mi ute. The reaction mixture was cooled and acidifi immediately. The crude acid was purified by r crystallization from 50% ethanol; m. p. 184 yield 8.2 Gm. (42%).

Anal.—Calcd. for $C_{10}H_8O_4$: C, 62.5; H, 4.1 Found: C, 62.12; H, 4.35.

Preparation of α-Ethoxy-β-(p-Chlorobenzoyl Ethyl Propionate.—This compound (No. 73) w a by-product in the esterification of β-(p-chlorobe zoyl)-acrylic acid by Procedure II. Boiling rang 156–158° (0.7 mm.).

Anal.—Calcd. for $C_{14}H_{17}O_4Cl$: C, 59.0; H, 5.9 Cl, 12.6. Found: C, 59.28; H, 5.81; Cl, 12.68.

Preparation of the Mercuric Acetate Addu (No. 69).—Sixteen grams of β-benzoyl acrylic ac and 32 Gm. of mercuric acetate were dissolved i 150 ml. of methanol, heated on the steam bath f two hours and then cooled. The product was r crystallized from dilute dioxane; m. p. 168° (dec. yield 16 Gm. (33%).

Anal.—Calcd. for $C_{14}H_{14}O_7Hg$; Hg, 40.54. Foun Hg, 40.61.

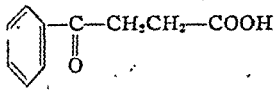
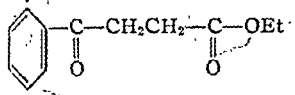
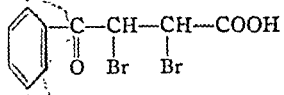
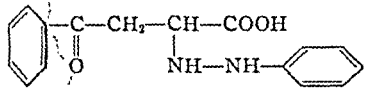
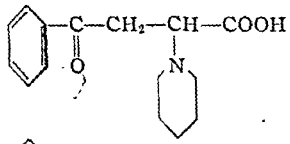
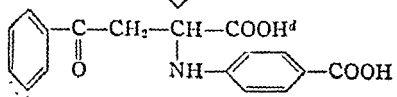
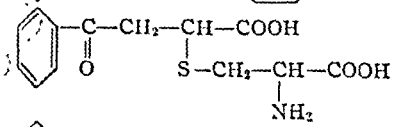
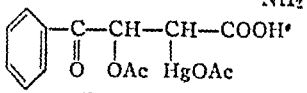
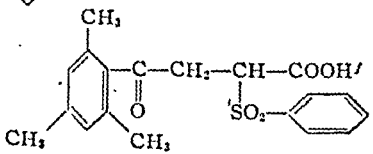
Effect of Cysteine Hydrochloride and p-Amin Benzoic Acid on Antibacterial Activity.—It was interest to determine if excesses of cysteine hydr chloride or p-amino benzoic acid could nullify th antibacterial potency of these compounds. Fro Table VI it can be concluded that these substance do not alter the inhibitory power of β-(o-hydroxy benzoyl)-acrylic acid.

Effect of Serum on Activity.—As is the case wit a host of other germicidal agents, our compound lose activity in the presence of serum. This is ev dent from the results in Table VII.

The Antibacterial Activity of the p-Carbethox Anilide of β-Mesitoyl Acrylic Acid against Severa Organisms.—Table VIII shows the inhibitory po tency of one of the most active compounds agains eight organisms. The test was carried out by th modified Raistrick method (see under Table II).

Toxicity.—The toxicity of the p-carbethoxy an lide of β-mesitoyl acrylic acid (No. 54) was deter

TABLE IV.—THE BACTERIOSTATIC ACTION OF SOME β-BENZOYL PROPIONIC ACID DERIVATIVES

No.	Compound	M. p. (Unc.), ° C.	B. p. (Unc.), °C.	Method of Prep.	Highest Dilution Causing Inhibition of Growth *S. aureus*	*E. typhosa*
62		114[a]	.....	I	.....*	.....
63		..	120 (0.8)[b]	II	.....	.....
64		148–52[c]	.....		.....	.....
65		199–202	.....	IV	.....	.....
66		156–60	.....	IV	.....	1:80,000
67		202	.....	IV	.....	.....
68		171	.....	IV	.....	.....
69		168	.....	..	1:640,000	1:640,000
70		93–5	.....	IV	.....	.....

(*Cont'd on p. 446*)

TABLE IV.—THE BACTERIOSTATIC ACTION OF SOME β-BENZOYL PROPIONIC ACID DERIVATIVES.—(*Cont'd from p. 445*)

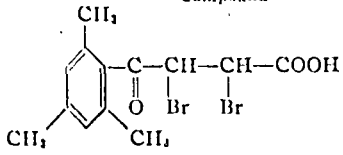
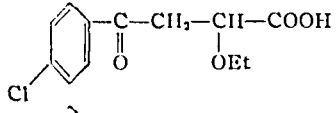
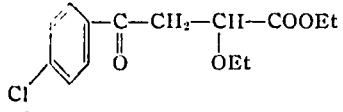
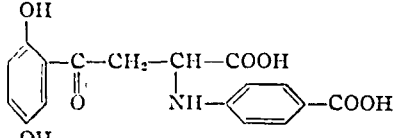
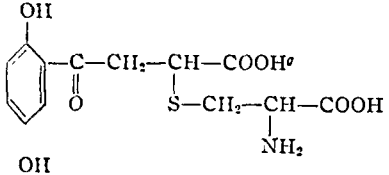
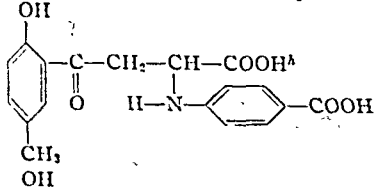
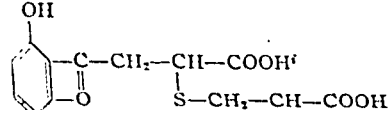
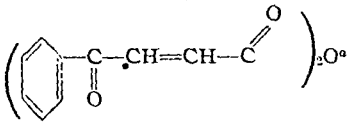
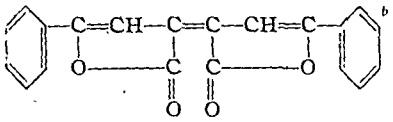
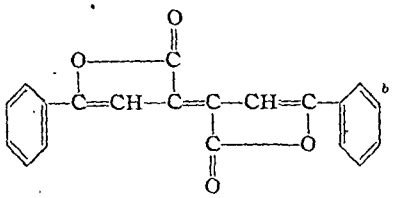
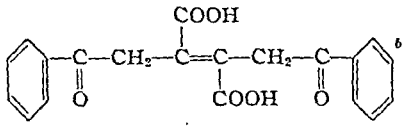
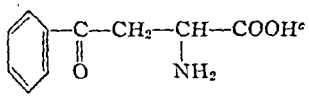
No.	Compound	N. p. (Unc.), °C	B. p. (Unc.), °C.	Method of Prep.	Highest Dilution Causing Inhibition of Growth *S. aureus*	*E. typhosa*
71		167–9	.....	..	.....	.....
72		140	.....	..	.....	.....
73		..	156–8 (0.7)	II	.....	.....
74		198	.....	IV	.....	.....
75		169–70	.....	IV	.....	.....
76		190	.....	IV	.....	.....
77		165	.....	IV	.....	.....

TABLE V.—THE BACTERIOSTATIC ACTION OF SOME MISCELLANEOUS COMPOUNDS

No.	Compound	M. P. (Unc)° C.	Highest Dilution Causing Inhibition of Growth *S. aureus*	*E. typhosa*
78		112–3	1:200,000	1:200,000
79		...	.....*	1:476,000
80		...	.....	1:408,000
81		...	.....	.....
82		...	.....	.....

* No activity in lowest dilution tested.
[a] Calcd. for $C_{20}H_{10}O_5$: C, 71.8; H, 4.2. Found: C, 72.12; H, 4.54. Structure proved by conversion to the theoretical amount of β-benzoyl acrylic acid.
[b] Prepared according to the method of Bogert and Ritter (24).
[c] Prepared according to the method described by Bougault (25).

TABLE VI.—BACTERIOSTATIC ACTION OF β-(*o*-HYDROXYBENZOYL)-ACRYLIC ACID IN THE PRESENCE OF CYSTEINE HYDROCHLORIDE OR P.A.B.A.

Antibacterial Agent (1:5000 in 10% EtOH)	Molar Equivalents of Cysteine HCl or P.A.B.A. to Antibacterial Agent	Highest Dilution Causing Inhibition of Growth *S. aureus*	*E. typhosa*
O.H.B.A.[a]	...	1:40,000	1:40,000
O.H.B.A. + Cysteine HCl	0.5	1:40,000	1:40,000
O.H.B.A. + Cysteine HCl	1.0	1:40,000	1:40,000
O.H.B.A. + Cysteine HCl	2.0	1:40,000	1:40,000
O.H.B.A. + Cysteine HCl	3.0	1:40,000	1:40,000
O.H.B.A. + P.A.B.A.	0.5	1:40,000	1:40,000
O.H.B.A. + P.A.B.A.	1.0	1:40,000	1:40,000
O.H.B.A. + P.A.B.A.	2.0	1:40,000	1:40,000

[a] β-(o-Hydroxybenzoyl)-acrylic acid.

mined in mice. Upon oral administration there was no death up to 1600 mg./Kg. Intraperitoneally, the LD_{50} was 75 mg./Kg.

FOOTNOTES TO TABLE IV

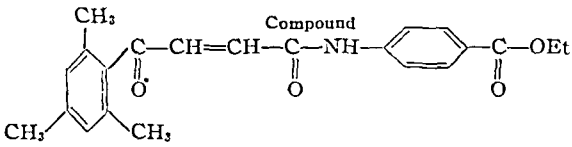
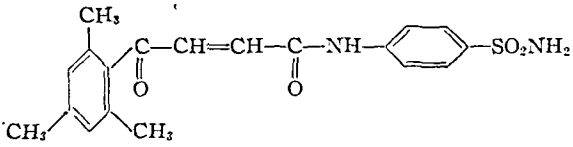
* No activity in the lowest dilution tested (1:10,000).
[a] M. p. reported, 116° (21).
[b] B. p. reported, 128° (2 mm.) (22).
[c] M. p. reported, 148° (23).
[d] Calcd. for $C_{17}H_{13}O_3N$: N. E., 157. Found: N. E., 159.
[e] Calcd. for $C_{18}H_{17}O_7Hg$: Hg, 40.5. Found: Hg, 40.61.
[f] Calcd. for $C_{19}H_{20}O_4S$: S, 8.9. Found: S, 8.8.
[g] Calcd. for $C_{18}H_{19}O_4NS$: S, 10.22. Found: S, 10.22, 10.35.
[h] Calcd. for $C_{18}H_{17}O_4N$: C, 62.97; H, 4.99; N. E., 171. Found: C, 62.65; H, 5.01; N. E., 167.
[i] Calcd. for $C_{18}H_{17}O_4NS$: S, 9.78. Found: S, 9.29, 9.35.

DISCUSSION

The acids listed in Table II afford an opportunity to make some generalizations about the relationship of chemical structure to antibacterial activity for this series of compounds. However, before attempting this it would be expedient to discuss the chemical properties of the parent compound, β-benzoyl acrylic acid, in order to gain a better insight into the possible mode of action of this series of antibacterial agents.

The β-aroyl acrylic acids contain an ethylenic linkage which shows remarkable ease of addition. Addition compounds of diazomethane, methanol,

TABLE VII.—EFFECT OF SERUM ON BACTERIOSTATIC ACTIVITY

Compound	Highest Dilution Causing Inhibition of Growth of *S. aureus*	
	No Serum	10% serum
	1:1,600,000	Inactive at 1:400,000
	1:800,000	Inactive at 1:400,000

and hydrogen chloride to β-(*p*-brombenzoyl)-acrylic acid have been prepared (26). In this laboratory the easy addition of cysteine and *p*-aminobenzoic acid to the β-benzoyl acrylic acid compounds has been observed. The resulting saturated ketones are no longer effective antibacterial agents. Thus it would appear that in conformance with the postulate of Geiger and Conn (1) these compounds owe their activity against bacteria to their ability to add some essential metabolite or enzyme system across the highly active α,β-unsaturated ketonic structure.

According to the work of Kohler (27), α,β-unsaturated ketones are converted into saturated ketones by 1,4-addition. In the case of β-benzoyl acrylic acid it was shown that HA reagents add to the conjugated system containing the keto group (28).

Although α,β-unsaturated carbonyl systems show a marked tendency to undergo conjugate addition there are many instances in which one of the multiple bonds functions independently. Kohler (29) has pointed out that nearly all reactions involving 1,2-addition to carbonyl are reversible, whereas the products formed by 1,4-addition (except with organometallic compounds) undergo rearrangement into saturated carbonyl compounds that are still capable of undergoing 1,2-addition. Under these conditions the products ultimately isolated do not represent the relative rates of 1,2- and 1,4-addition but merely the relative stability of the substances or the position of the equilibrium in the particular environment.

The following reactions could conceivably occur between an HA compound (in this case HA = some bacterial enzyme system or some essential metabolite) and the β-aroyl acrylic acids.

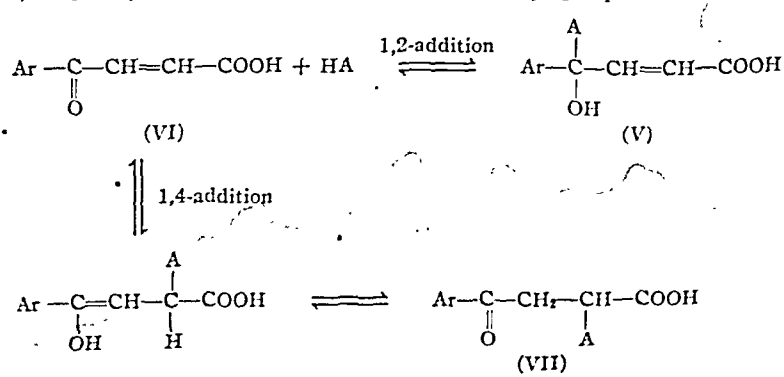
TABLE VIII.—THE BACTERIOSTATIC ACTION OF THE *p*-CARBETHOXY ANILIDE OF β-MESITOYL ACRYLIC ACID

Organism	Highest Dilution Causing Inhibition of Growth
Staphylococcus aureus No. 2092	1:1,000,000[a]
Staphylococcus albus No. 2	1:1,000,000[a]
Streptococcus hemolyticus No. 2	1:2,000,000[b]
Streptococcus viridans No. 4	1:5,000,000[b]
Diplococcus pneumoniae Type II	1:5,000,000[b]
Hemophilus influenzae No. 2	1:2,000,000[b]

[a] *Medium.*—F. D. A. broth.
[b] *Medium.*—Beef infusion broth.

Of the acids listed in Table II only five have an activity over 1:100,000 against *S. aureus* and of these only the triethyl benzoyl acrylic acid (No. 4) shows a similar efficacy against *E. typhosa*. It is difficult to pick out any characteristic structural feature in the substituted benzoyl acrylic acids, which accounts for the enhancement in antibacterial activity. However, in the case of β-mesitoyl acrylic acid (No. 3) and β-(2,4,6-triethyl benzoyl)-acrylic acid (No. 4) it would appear that steric hindrance might play a role. Furthermore, one is tempted to attribute the enhancement to a purely geometric effect because the larger alkyl group is much more effective.

The presence of the alkyl groups in both ortho positions to the unsaturated side chain would inactivate the carbonyl group. Thus, for example, the keto group of acetomesitylene is inactive toward the usual carbonyl reagents (30). As the activity of the carbonyl group decreases the amount of 1,4-



addition increases (31), consequently in the case of the sterically hindered β-aroyl acrylic acids the amount of the unstable adduct (V) formed by 1,2-addition would be negligible. This would result in a rapid formation of the more stable adduct (VII) and thus account for the greater antibacterial activity.

With the other three active acids (Nos. 24, 26, and 27) it is difficult to arrive at any definite relationship between structure and activity. However, it may be that greater solubility in the bacterial cell accounts for their inhibitory power in higher dilution.

The esters and amides are much more effective than the corresponding acids. Here again it could be a matter of better solubility in the bacterial cell.

From the results in Table III it would appear that there is no advantage in replacing the benzene ring by a thiophene, carbazole, or dibenzofuran ring.

All the compounds listed in Table IV, with the exception of two, show no inhibitory properties in the lowest dilution tested. The activity of the piperidine adduct (No. 66) is difficult to explain. However, the mercuric acetate derivative (No. 69) most likely owes its antibacterial action to the mercuric acetate group. This is in conformance with the well-known proficiency of the organomercurials as bacteriostatic agents.

The lack of activity in the other β-benzoyl propionic acid derivatives is evidence for the essentiality of the α,β-unsaturated system.

The first three compounds in Table V (Nos. 78, 79, and 80) show some promise. We are unable to explain the lack of activity against *S. aureus* by the latter two.

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The esters and amides are more effective than the corresponding acids.

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The bacteriostatic potency of two of the most effective compounds was greatly reduced in the presence of serum.

Saturation of the ethylenic linkage in the side chain brings about a loss in antibacterial activity.

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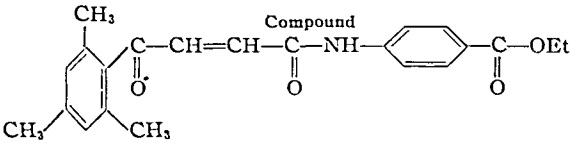
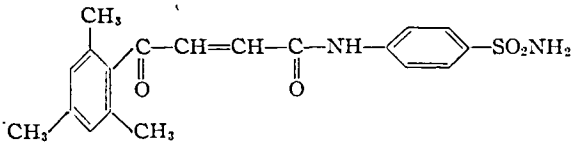
	1:1,600,000	Inactive at 1:400,000
	1:800,000	Inactive at 1:400,000

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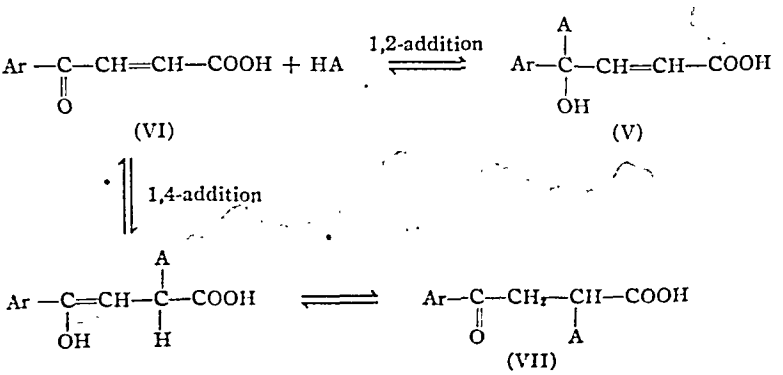

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Pharmacognostical and Chemical Studies of Indian Belladonna, *atropa acuminata* Royle ex Lindley*

By HEBER W. YOUNGKEN† and WILLIAM E. HASSAN, JR.‡

Comparative gross anatomical and histological studies are reported on the roots, stems, leaves, flowers, and fruits of *Atropa acuminata* and of *Atropa Belladonna*. A chemical study of the alkaloidal content of both species is reported in which it is shown that Indian Belladonna possesses a higher alkaloidal content than the European species.

In the BritishPharmacopœia of 1932, add. 7 (1), along with *Atropa Belladonna*, we find *Atropa acuminata* mentioned as an additional source of Belladonna. The latter is an Indian species and has been gathered in large amounts for use in the British Commonwealth of Nations. A number of pharmacognosists and chemists have reported on various phases of Indian Belladonna but the reports have been scattered and some of the literature pertaining to the taxonomy and alkaloidal constituents has been chaotic. The purpose of this paper is to straighten out this confusion by attempting to confirm some of the results hitherto reported and adding new facts concerning the structure, constituents, and taxonomy of Indian Belladonna. Representative specimens consisting of herbarium sheets and preserved materials have been permanently deposited in the herbarium of the Massachusetts College of Pharmacy.

TAXONOMY

The taxonomic history of this plant may be traced back to the middle of the eighteenth century. Two names have been applied to it, *Atropa acuminata* Royle and *Atropa lutescens* Jacquemont but both of these are equated to *Atropa Belladonna* L. in the Index Kewensis. The name *Atropa acuminata* was used by Royle in his *Illustrations of the Botany of the Himalaya* without a description and is a *nomen nudum*.

Atropa lutescens Jacquemont has been used by Aitchinson (2) in his account of the flora of the Kurrum Valley, by Watt (3) in his *Dictionary of the Economic Products of India*, and by several other pharmaceutical writers. There is no mention of an *Atropa* in Jacquemont's *Voyage Dans L'Inde Botanique*, 1884, and he does not seem to have validated his manuscript name elsewhere by a description.

Clark (4) cites *Atropa lutescens* Jacquemont together with *Atropa acuminata* Royle as synonymous with *Atropa Belladonna* L. in Hook. f., *Flora of British India*. (4), though *Atropa acuminata* is validated much earlier by Lindley in the *Journal of the Horticultural Society of London* (5) where a brief description is given of plants raised in the Society's garden from seeds sent by Capt. William Munroe in 1845.

Tschirch, in his *Handbuch der Pharmakognosie*, 1923 (6), uses the combination *Atropa acuminata* Meirs. In this he is followed by Hegi in *Flora von Mittel-Europa*, 1927, but Meirs himself attributes the name to Royle. The synonymy and the relevant literature pertaining thereto are as follows:

Atropa acuminata Royle
"Bot. Himal.," 279, (1839), *nomen nudum.*
Atropa lutescens Jacquemont, *nomen nudum.*
Atropa acuminata Royle ex Lindley
J. Hort. Soc. (*London*), I, 306(1846).
Atropa acuminata Royle ex Meirs
J. Bot., I, 138(1849) and "Illust. S. Amer. Plants," ii, app. 5, 5, T76, 1857.
Atropa acuminata, Meirs
Tschirch, A., "Handbuch der Pharmakognosie," III (i), 268, 1923.
Atropa lutescens, Jacquemont ex Clarke
Hooker fil. "Flora of British India, Vol. IV, 241(1885).
Atropa acuminata Meirs ex Hegi
"Flora von Mittel-Europa," Vol. V (4), 2565, (1927).

It is quite evident that the nomenclature for Indian Belladonna has been chaotic.

MATERIALS

The materials for this investigation consisted of the entire plants of *Atropa Belladonna* L. and *Atropa acuminata* Royle ex Lindley. The seeds of the European variety were gathered from the fruits of authentic plants of *Atropa Belladonna* grown in the College Medicinal Plant Garden. Those of the Indian variety were obtained from Dr. A. F. Sievers of the United States Department of Agriculture Station, Beltsville, Maryland.

The seeds were first planted in flats and the seedlings kept in a local greenhouse until they were transplanted into individual flower pots. When they were large enough, some of the plants were again transplanted into the College Medicinal Plant Garden in Jamaica Plain, Boston, Massachusetts, and others were planted at Brockton, Massachusetts.

Specimens of the roots, stems, leaves, and flowers were gathered at various times during their growth, examined macroscopically and microscopically, and the surplus preserved materials have been deposited in the Herbarium of the Massachusetts College of Pharmacy.

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Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.
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‡ Fellow in Pharmacognosy, Massachusetts College of Pharmacy.

DESCRIPTION OF THE PLANT

Indian Belladonna (Fig. 1) as grown in Boston, Massachusetts, is a bushy perennial herb with dichotomously branched stems. The plant attains an average height of 3 to 4 ft. The leaves (Figs. 1 and 2) vary in color from green to olive-green and measure up to 30 cm. in length and up to 12 cm. in width. They are oblong-elliptical, tapering gradually at both the apex and the base, whereas the leaves which are located close to the flowering tops are more pointed at the apex and less tapering at the base. On the flowering branches, the leaves occur in pairs, nearly opposite, the lowest one having a single flower in its axil. The slightly higher one exhibits a secondary branching system with 2 to 4 flowers together in a bracteate cyme. The angle which the second and third lateral veins of the leaves make with the midrib is usually from 30 to 40 degrees.

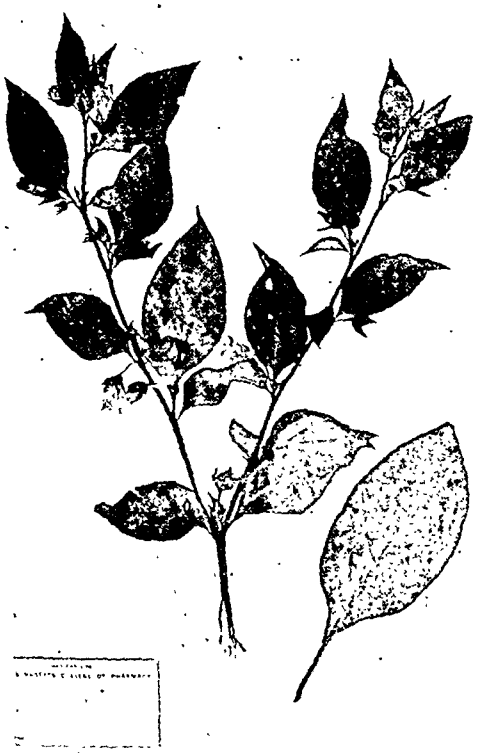

Fig. 1.—*Atropa acuminata* Royle ex Lindley. Portion of plant grown in medicinal plant garden of the Massachusetts College of Pharmacy showing leaf and flowering branches and a larger leaf from another branch of the same plant. × 1/3.

The flowers occur either solitary, or in groups of 2 to 4, in bracteate cymes. They are axillary and drooping and possess a calyx which is deeply 5-cleft and a yellow to greenish yellow, campanulate to tubular-campanulate corolla with 5 broad, spreading, recurved lobes. There are 5 epipetalous stamens, an exserted style, and a knob-like stigma. The ripe fruit is a sub-globular black berry with a persistent calyx.

The rootstock is hard and woody, from 3 to 5 cm. in width, and the first year root, which is comparatively small in bulk, does not exceed 2 cm. in width. Upon drying, the root becomes wrinkled longitudinally. The fracture is tough and does not always break with a short mealy fracture as is characteristic of the root of the European Belladonna.

PALISADE RATIO OF INDIAN AND EUROPEAN BELLADONNA

Zörnig and Weiss (7) in their paper on certain compositaceous leaves stated that the average number of palisade cells beneath an upper epidermal cell was diagnostically valuable. They also stated that the number of palisade cells per unit area increased successively from the base of the leaf to the upper portion of the apex but since there was now a corresponding diminution in the area of the epidermal cells, the ratio remained almost constant.

Wallis and Dewar (8), later, in their study of the

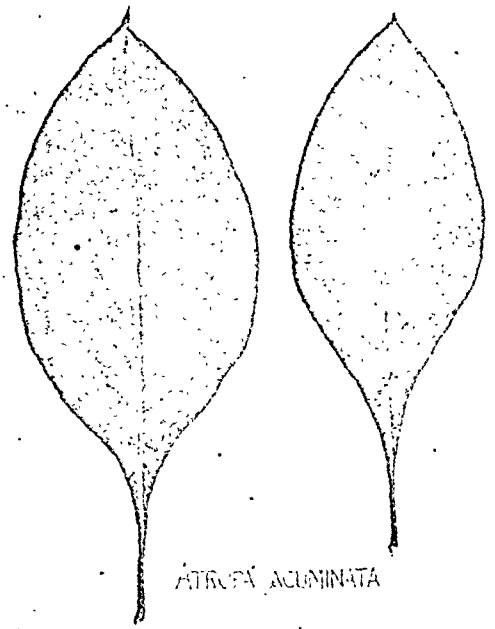

Fig. 2.—Leaves of *Atropa acuminata* Royle ex Lindley. × 1/2.

comparative anatomy of the Barosmas, concluded that variation in the palisade ratio is independent of the position on the leaf. Furthermore, they were the first to use the term "palisade ratio" stating in their publication that the total number of palisade cells beneath four upper epidermal cells, when divided by four, gave the recorded figure for the palisade ratio, this being the term and method employed in this paper to express the average number of palisade cells beneath an upper epidermal cell.

Wallis and Forsdike (9), in their investigation of the palisade ratio of *Atropa Belladonna Scopolia*

carniolica, and *Solanum nigrum*, concluded that the palisade ratio does not change with the age of the leaf, the habitat or from year to year within any definite species.

Dewar (10–12) has reported on the palisade ratios of *Digitalis Thapsi*, *D. lutea* , and *D. lanata*.

Feinstein and Slama (13) found that the palisade ratio could be used in differentiating the leaves of *Atropa Belladonna* from those of official Stramonium, Hyoscyamus, and Digitalis.

George (14), in his study of the ratio of the Indian and the European varieties of Belladonna, concluded that *Atropa acuminata* had a palisade ratio which falls within the range of 6.9 to 9.2, whereas *Atropa Belladonna* falls within the range of 5.0 to 7.0.

From the foregoing discussion, it seemed possible that the palisade ratio might prove to be of pharmacognostic value in aiding in the differentiation of these two species of Belladonna.

TABLE I.—PALISADE RATIOS OF ATROPA BELLADONNA LEAVES

Location	No. 1	No. 2	No. 3	No. 4	No. 5	Location, Av.
Base	7.7	7.0	5.0	4.5	5.2	
	8.0	5.5	4.5	5.2	4.5	
	7.0	6.0	6.0	5.0	4.8	
	7.5	6.5	5.5	4.5	5.2	
	8.0	5.2	5.2	6.0	5.0	
Av.	7.6	6.0	5.2	5.0	4.9	5.7
Margin	8.5	6.5	5.0	6.5	5.8	
	9.0	6.0	5.2	5.5	6.7	
	8.8	6.7	5.5	5.7	5.5	
	9.2	5.5	5.7	5.0	5.5	
	9.0	6.0	4.5	5.4	5.0	
Av.	8.9	6.1	5.2	5.6	5.7	6.3
Center	7.0	6.5	5.5	6.2	6.7	
	7.8	6.2	5.5	6.0	6.2	
	7.2	6.5	6.5	5.5	5.5	
	7.0	6.0	6.0	5.5	5.2	
	7.2	7.0	5.8	6.0	6.0	
Av.	7.2	6.4	5.8	5.8	6.0	6.2
Apex	7.0	7.3	5.5	6.5	5.2	
	7.7	7.2	5.0	5.5	5.0	
	6.5	6.5	5.2	6.0	6.0	
	7.5	6.6	5.5	5.5	5,5	
	6.5	7.0	6.0	5.5	5.0	
Av.	7.1	6.9	5.4	5.8	5.3	6.1
Av. per leaf	7.7	6.3	5.4	5.5	5.5	

Notes: 1. Average palisade ratio for the species 6.1.
2. Lowest recorded palisade ratio 4.5.
3. Highest recorded palisade ratio 9.2.
4. Mean average 6.9.

The method followed was to cut out pieces 2–3 mm. square, with the aid of a sharp blade, from the apex, margin, center, and base. These segments were then placed in a clearing solution which consisted of 50 Gm. of chloral hydrate dissolved in 20 cc. of water and gently heated upon a water bath for approximately fifteen minutes. The material was then removed and mounted in Berlese's Modified Mountant. In each determination, the number of palisade cells under any four adjacent epidermal cells, when divided by four, represented the palisade ratio and in no case was any palisade cell included where more than one-half of its area lay outside of the epidermal cell.

The results of this study are recorded in Tables I and II which are constructed to show the palisade ratio for each leaf, for the specific location, and for the species.

VEIN ISLET NUMBER DETERMINATION

Eames and MacDaniels (15) define a vein islet as "the ultimate divisions of the conducting strands completely or partially encircling minute areas of the photosynthetic tissues with which they are in close contact."

The term "Vein Islet Number" was coined by Levin who defined it as "the number of vein islets in a square millimeter of leaf tissue."

TABLE II.—PALISADE RATIOS OF ATROPA ACUMINATA LEAVES

Location	No. 1	No. 2	No. 3	No. 4	No. 5	Location, Av.
Base	8.5	8.0	5.0	7.5	8.2	
	8.0	7.5	5.8	7.0	7.5	
	7.7	7.0	6.5	7.6	7.8	
	7.5	7.5	6.5	8.0	8.0	
	8.0	7.7	5.5	8.1	8.0	
Av.	7.7	7.5	5.8	7.6	7.9	7.3
Margin	11.5	8.5	6.0	8.2	8.2	
	10.2	8.5	6.8	8.0	8.9	
	10.0	9.0	7.5	8.6	9.8	
	9.8	8.6	8.0	9.0	8.5	
	9.5	9.0	8.2	9.5	8.8	
Av.	10.2	8.7	7.3	8.4	8.8	8.7
Center	9.0	8.5	8.5	8.2	8.7	
	9.8	8.2	7.5	8.0	8.2	
	8.8	8.5	9.5	9.5	7.5	
	8.8	7.0	7.0	8.5	6.6	
	8.4	8.0	7.8	9.0	7.0	
Av.	8.9	8.0	8.0	8.6	7.6	8.2
Apex	12.4	9.0	8.8	9.2	8.8	
	11.6	8.5	7.8	8.6	8.5	
	11.1	8.7	9.2	9.1	7.8	
	10.8	8.3	8.0	8.7	7.9	
	10.7	8.2	8.8	8.5	8.2	
Av.	11.6	8.5	8.3	8.5	8.2	9.0
Av. per Leaf	9.6	8.2	7.3	8.3	8.1	

Notes: 1. Average palisade ratio 8.3.
2. Lowest palisade ratio 5.0.
3. Highest palisade ratio 12.4.

In our examination, a method proposed by Zufall and Burlage (16) was employed, using a slight modification. Pieces of the leaf material approximately 4 mm. square were cut from the apex, center, margin, and base. After clearing with chloral hydrate solution, the sections were mounted on glass slides in the clearing medium and examined microscopically.

The area of the vein islet may be determined by counting the number of vein islets within the field of the 16 mm. objective. The number of vein islets

within the field divided by the area of the field, in mm., gives the average area of the vein islet.

The 16-mm. objective gave a field whose area was 1.98 sq. mm. This is close enough to the whole number 2, so that the "Vein Islet Number" can be easily calculated. The vein islets in a field are counted and the "Vein Islet Number" is obtained by dividing this figure by two. The data obtained are recorded in Tables III–VII.

TABLE III.—VEIN ISLET NUMBER DETERMINATIONS OF THE LEAF APEX OF ATROPA ACUMINATA[a]

Leaf No.	Field No.	Av. V. I. N.	Leaf Position, Av.
1	1	14	
1	2	11	
1	3	12	
1	4	12	14.7
2	1	15	
2	2	11	
2	3	13	
2	4	10	12.2
3	1	12	
3	2	10	
3	3	12	
3	4	6	10.0
4	1	10	
4	2	9	
4	3	9	
4	4	7	8.7
5	1	10	
5	2	9	
5	3	9	
5	4	8	9.0

[a] See Fig. 2.

TABLE IV.—VEIN ISLET NUMBER DETERMINATIONS OF THE LEAF CENTER OF ATROPA ACUMINATA

Leaf No.	Field No.	Av. V. I. N.	Leaf Position, Av.
1	1	11	
1	2	10	
1	3	10	
1	4	8	9.7
2	1	11	
2	2	10	
2	3	12	
2	4	10	10.7
3	1	10	
3	2	10	
3	3	10	
3	4	9	9.7
4	1	10	
4	2	10	
4	3	9	
4	4	9	9.5
5	1	10	
5	2	9	
5	3	8	
5	4	9	9.0

STOMATAL INDICES

We have investigated the stomatal indices of the Indian and the European varieties of Belladonna using the formula advocated by Salisbury (17) and the procedure used by George (19) for Senna.

The results of our investigation are in accord with those obtained by Rowson (18) in his investigation on the English-grown plant. The results of our investigation are as follows:

Upper surface of Indian Belladonna....	3.4
Upper surface of European Belladonna..	2.9
Lower surface of Indian Belladonna....	17.6
Lower surface of European Belladonna..	17.6

TABLE V.—VEIN ISLET NUMBER DETERMINATIONS OF THE LEAF MARGIN OF ATROPA ACUMINATA

Leaf No.	Field No.	Av. V. I. N.	Leaf Position, Av.
1	1	13	
1	2	14	
1	3	13	
1	4	14	13.5
2	1	13	
2	2	12	
2	3	11	
2	4	10	11.5
3	1	10	
3	2	13	
3	3	12	
3	4	10	11.2
4	1	12	
4	2	10	
4	3	12	
4	4	11	11.2
5	1	12	
5	2	11	
5	3	12	
5	4	10	11.2

TABLE VI.—VEIN ISLET NUMBER DETERMINATIONS OF THE LEAF BASE OF ATROPA ACUMINATA

Leaf No.	Field No.	Av. V. I. N.	Leaf Position, Av.
1	1	9	
1	2	7	
1	3	7	
1	4	7	7.5
2	1	8	
2	2	12	
2	3	9	
2	4	10	9.7
3	1	10	
3	2	7	
3	3	8	
3	4	7	8.0
4	1	10	
4	2	7	
4	3	8	
4	4	7	8.0
5	1	10	
5	2	10	
5	3	11	
5	4	10	10.0

TABLE VII.—SUMMARY OF V. I. N. DETERMINATIONS

Leaf av. no. 1.....	11.3
2.....	13.5
3.....	10.2
4.....	9.3
5.....	9.8
Species av.....	10.0

A COMPARATIVE STUDY OF THE FLORAL PARTS OF INDIAN AND EUROPEAN BELLADONNA

The flowers for this study came from the first year plant soon after the opening of the calyx and corolla. The measurements were made upon 25 freshly gathered flowers with a metric rule.

The calyx of the Indian variety is green to yellowish green and glandular-pubescent. The sepals are joined to form a cup from 0.4 to 0.7 cm. long. The lobes of the calyx measure from 1.0 to 2.1 cm. in length and are triangular to ovate-triangular, the apices are acuminate, and the entire margin recurved. Estivation is valvate to imbricate.

The calyx of the European variety compares favorably to the above description with the exception that the calyx here is a true green at all times. The sepal cup measures from 0.3 to 0.6 cm. and the lobes of the calyx are from 1.0 to 1.5 cm. in length.

The corolla of *Atropa acuminata* is campanulate to tubular campanulate and from 1.5 to 2.8 cm. in length and from 0.5 to 1.3 cm. in width at the mouth. The lobes are from 0.3 to 0.7 cm. in length and are semielliptical with entire rolled margins. The corolla is bright yellow to greenish yellow in color with purplish veins in the lower portion and a dull brownish margin around the mouth.

The European variety exhibits a campanulate corolla measuring from 2.4 to 3.3 cm. in length and from 1.1 to 1.4 cm. in diameter at the mouth. The lobes vary slightly in that they measure from 0.4 to 0.7 cm. in length. The corolla is a vivid purple merging into a pale green where it enters the calyx. The under surface is velvety in the upper part and glossy where green. Pigmentation of the veins is a dark purple.

The style of the Indian variety is filiform and solid, and ranges in length from 1.6 to 2.0 cm. It rises from the apex of the ovary and varies in color from yellowish green to yellow-white. It is slightly arched and is terminated by a capitate, obscurely bilobed stigma which is yellow-green in color and quite fleshy.

The style of European Belladonna is longer than that of the Indian variety, the length varying from 2.3 to 2.5 cm.

The stamens of the Indian variety are epipetalous and measure from 1.0 to 1.5 cm. in length. Greenish yellow filaments are adherent to the base of the corolla for a very short distance over the point of adhesion. Numerous long trichomes grow from the corolla and the filaments. The filaments are hooked beneath the anthers which are about 0.2 cm. long, dorsifixed, and dehiscing introrsely by two longitudinal splits; the walls contract to expose a mass of pale yellow pollen.

The stamens of the European Belladonna are epipetalous and measure 1.5 to 2.0 cm. in length, and the anthers are approximately 0.3 cm. in length.

The ovary of the Indian variety is sphero-conical and about 0.3 cm. in length and from 0.3 to 0.4 cm. in diameter at the widest part. It is superior, bicarpellary, and bilocular with two longitudinal depressions externally, corresponding in position to the edges of the septum. The nectary is in the form of an annular swelling at the base. Placentations seem to be axile. The placentae and the small ovules completely filled the loculi. No significant variation was noted between the ovaries of the two plants.

STEM HISTOLOGY OF ATROPA ACUMINATA

Sections of the stem 2.5 to 3.0 mm. in diameter (Fig. 3) presented the following microscopical characteristics.

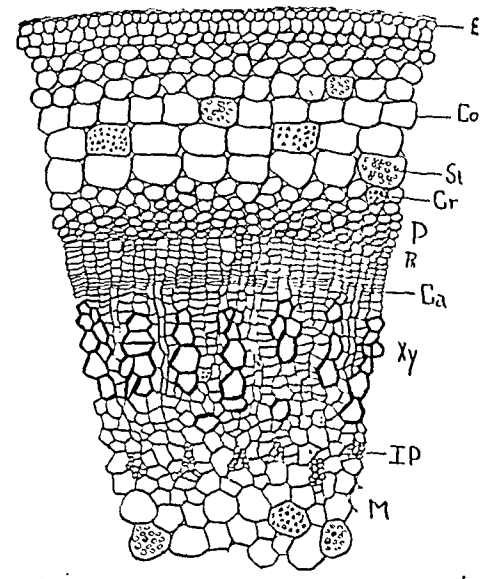

Fig. 3.—*Atropa acuminata.* Transverse section of stem. *Ep*, epidermis; *Co*, cortex; *St*, starch; *Cr*, microcrystals; *P*, pericycle; *Ph*, phloem; *Ca*, cambium: *Xy*, xylem; *IP*, internal phloem; *M*, pith.

The epidermis consisted of small square to slightly tangentially elongated epidermal cells, as viewed in cross section, the cells polygonal to irregular-shaped in surface section, with a striated cuticle and beaded vertical walls, and showing numerous uniseriate, nonglandular hairs, many up to 5-celled, a few up to 6-celled, also numerous glandular hairs with up to 5-celled, uniseriate stalks and small subspherical to spherical heads. Numerous stomata of the solanaceous type were present, the stomates being predominantly oval in shape.

The cortex consisted of approximately 6 layers of chlorophylloid collenchyma.

The pericycle was narrow and consisted of 2 or 3 layers of starch and microcrystal-bearing parenchyma in which were scattered pericyclic fibers with thin, nonlignified walls and broad lumina.

The sections showed many bicollateral fibrovascular bundles with narrow external and internal phloem and broader xylem zones, the xylem exhibiting narrow wood rays and wood wedges, the latter consisting chiefly of tracheary tissue and a limited number of wood fibers.

The pith is composed of richly protoplasmic, pitted parenchyma, many of the cells containing masses of microcrystals.

Sections of the stem 10 mm. or more in diameter exhibited an epidermis which differed from the

younger stems in showing relatively few hairs, larger epidermal cells, the latter with nonbeaded or indistinctly beaded vertical walls, many containing pink to purple anthocyanin. The cortex is broader, mostly parenchymatous with larger cells. The pericyclic fibers are lignified and thicker-walled. The xylem wedges are deeper than in younger portions of the stem and show a considerable increase of wood fibers. The internal phloem exhibits larger islets of sieve. The tracheae and the trachieds are of the simple pitted, bordered pitted, reticulate, and spiral types occurring in the primary xylem. The pith is broad consisting of pitted parenchyma, the cells of which are mostly larger than in young stems and, in sections 10 mm. in diameter, show beginning disintegration in the central area. A number of scattered pith cells contained microcrystals. Sections of the stem cut in midsummer showed a green pith, numerous chloroplastids, and small starch grains. The medullary rays possess lignified walls in older stem parts.

COMPARATIVE HISTOLOGY OF THE ROOTS OF THE INDIAN AND EUROPEAN VARIETIES OF BELLADONNA

During the course of this comparative histology, the work of Melville (20) was consulted frequently and the figures stated here are within or relatively close to the range of his findings.

The cork of *Atropa acuminata* is similar to that of *Atropa Belladonna* except that the cells in surface view are larger. The dimensions from the measurements of approximately 200 cells are: length minimum 30 μ, maximum 200 μ, with the majority lying between 50 and 130 μ. Melville states that the average length per cell is 95 μ. The width showed a minimum of 20 μ and a maximum of 86 μ with the majority lying between 32 and 56 microns. The stated general width average per cell was 44 μ.

For *Atropa Belladonna* L., the dimensions for a similar number of measurements were such that the average for the length was 72.5 μ and for the width 39.5 μ.

Melville (20) in his investigation stated that the cork cells per sq. mm. calculated from counts of 25 fields of about 0.1 sq. mm. were for the Indian root a minimum of 167 to a maximum of 400, the majority numbering between 225 and 345. The mean is 285. A similar count on *Atropa Belladonna* yielded a mean of 470. A statistical analysis of the figures shows that the mean of 10 such counts will fall within 347 to 593 cells per sq. mm. for *Atropa Belladonna* and within the range of 227 and 344 cells per sq. mm. for *Atropa acuminata*. One may then consider that if the mean of 10 such counts is taken, a value above 347 indicates *Atropa Belladonna* and below, *Atropa acuminata*.

The phellodermconsists of about 10 layers of cells differing from those of *Atropa Belladonna* in that some contain a reddish brown resinous substance in addition to the starch and crystals.

The secondary phloem consists of up to 50 layers of cells, mainly starch-and microcrystal-bearing parenchyma and radial groups of sieve tissue similar to that of *Atropa Belladonna*. On the outer border are a few isolated fibers with associated groups of collapsed sieve tissue; scattered throughout the [illegible] walls are striated, thick, and show simple pitting. The fibrous cells appear as axially elongated irregular prisms, with the walls moderately thick and with a few relatively small simple pits. They average approximately 300 μ in length and 28 μ in diameter.

The cambium consists of up to 3 layers of collapsed cells, and is similar to that of *Atropa Belladonna*.

The secondary xylem in young roots is composed of starch- and crystal-bearing parenchyma with strands of up to 10 vessels and associated tracheids, fibers, and lignified parenchyma. The strands located close to the cambium are radially arranged forming a hollow cylinder, but are more scattered as they approach the center, an arrangement similar to that of *Atropa Belladonna*.

The primary xylem is a central, solid, di-arch strand similar to that of *Atropa Belladonna*.

The medullary rays at the cambium average up to 7 per mm.; in tangential view they are elongate, spindle-shaped and up to 20 cells high and 1 to 5 cells wide. The cells are similar to those of *Atropa Belladonna* or are often more axially elongated. The average length ranges between 80 and 180 μ.

Starch occurs abundantly in all of the parenchymatous tissues, the individual grains being similar to those of *Atropa Belladonna*. The diameter of 100 measured grains did not exceed 15.0 μ. More measurements are contemplated.

Sphenoidal microcrystals occur in all of the parenchymatous tissues of both species.

A COMPARATIVE STUDY OF THE BIOSYNTHESIS OF HYOSCYAMINE

Cromwell (21), during his studies on the biosynthesis of various Solanaceous alkaloids, discovered that alkaloids in general are synthesized from prod-

TABLE VIII.—ATROPA ACUMINATA

Solution Injected	Leaf Control	Leaf Experimental	Root Control	Root Experimental
Potassium Nitrate	0.342	0.335	0.525	0.530
Ammonium Sulfate	0.340	0.366	0.528	0.538
Glycine	0.338	0 357	0.524	0.548
Arginine	0.345	0.379	0.525	0.544
Putrescine[a]	0.346	0.483	0.528	0.586
Methylamine[a]	0.341	0.349	0.526	0.548
Dimethylamine[a]	0.342	0.350	0.524	0.545
Trimethylamine[a]	0.345	0.356	0.525	0.539

TABLE IX.—ATROPA BELLADONNA

Solution Injected	Leaf Control	Leaf Experimental	Root Control	Root Experimental
Potassium Nitrate	0.320	0.324	0.515	0.520
Ammonium Sulfate	0.322	0.348	0.518	0.527
Glycine	0.328	0.342	0.514	0.538
Arginine	0.325	0.358	0.515	0.534
Putrescine[a]	0.325	0.452	0.518	0.563
Methylamine[a]	0.320	0.331	0.516	0.538
Dimethylamine[a]	0.324	0.326	0.514	0.535
Trimethylamine[a]	0.325	0.342	0.515	0.529

[a] Dihydrochloride.

The values given represent the average of 3 injections on separate plants in the seventh month of their first year's growth.

Figures represent Gm. of alkaloid per 100 Gm. of dry weight.

ucts of carbohydrate and protein breakdown. In a report published in 1943, Cromwell (21) showed that the alkaloid hyoscyamine could be biosynthesized in *Atropa Belladonna* by use of the various amino acids and related amines.

Using Cromwell's method, the European and the Indian varieties of Belladonna were subjected to the various amino salts and other chemicals for the purpose of comparing the biosynthesis of the alkaloid hyoscyamine in the two species.

A solution containing 2% glucose and 0.25% of the active substance was placed in a one-liter aspirator flask approximately 2.5 to 3.0 ft. above the plant and the solution conveyed to the cut end of the stem through a rubber tubing provided with a screw clip to serve as a shut-off. With good transpiration, as much as 100 cc. of the solution passed through the plants in a single day. The injection was allowed to carry on for a week. The plant was then cut, dried at room temperature, and chemically assayed.

The comparative results of this experiment are given in Tables VIII and IX.

CONSTITUENTS

When the British Pharmacopœial Commission admitted Indian Belladonna to the British Pharmacopœia to serve as an alternative to the European variety in galenical preparations, a controversy arose as to the reliability of the official method of assay. This method did not insure more than the partial removal of the known volatile bases, therefore causing a variation in the final alkaloidal determination.

Evers and Caines (22) and Markwell and Walker (23), through numerous chemical assays, have concluded that *Atropa acuminata* contained a higher per cent of alkaloid and a far greater per cent of volatile bases.

The volatile bases have therefore been subjected to chemical analysis by various authors. It has been stated that the base contains a variable amount of tropine, pyridine, N-methylpyrroline, N-methylpyrrolidine, and tetra-methyldiaminobutane.

The following method of assay was employed in the chemical investigation of the Belladonnas:

TABLE X.—ATROPA ACUMINATA BERRIES

Sample No.	Drying Method	Temp., ° C.	Wt., Gm.	Alkaloid, %
1	Infrared	145	25	0.060
2	Infrared	145	25	0.055
3	Infrared	145	25	0.058
4	Infrared	55	25	0.296
5	Infrared	55	25	0.308
6	Infrared	55	25	0.305
7	Infrared	55	25	0.305
8	Infrared	55	25	0.306
9	Natural	22	25	0.309
10	Natural	22	25	0.306
11	Natural	22	25	0.303
12	Natural	22	25	0.306

Samples 1, 2, and 3 were dried under two infrared lamps, one set 10 inches above the sample and the other 10 inches below. Drying was allowed to go on for one hour.
Samples 4, 5, 6, 7, and 8 were dried under one infrared lamp placed at a distance of 24 inches above the fruits. Drying was allowed to continue for a period of two hours.
Samples 9, 10, 11, and 12 were spread out and allowed to dry at room temperature. The length of time for drying was approximately six to eight weeks.

TABLE XI.—ATROPA BELLADONNA BERRIES

Sample No.	Drying Method	Temp., ° C.	Wt. Gm.	Alkaloid, %
1	Infrared	145	25	0.051
2	Infrared	145	25	0.050
3	Infrared	145	25	0.051
4	Infrared	55	25	0.286
5	Infrared	55	25	0.295
6	Infrared	55	25	0.296
7	Infrared	55	25	0.296
8	Infrared	55	25	0.296
9	Natural	22	25	0.300
10	Natural	22	25	0.298
11	Natural	22	25	0.298
12	Natural	22	25	0.298

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Samples 9, 10, 11, and 12 were spread out and allowed to dry at room temperature. The length of time for drying was approximately six to eight weeks.

TABLE XII.—BERRY AND CALYX OF ATROPA ACUMINATA[a]

Sample No.	Temp., ° C.	Wt. Gm.	Alkaloid, %
1	22	25	0.55
2	22	25	0.51
3	22	25	0.50
4	22	25	0.50
5	22	25	0.49
6	22	25	0.46
7	22	25	0.51
		Av.	0.503

TABLE XIII.—BERRY AND CALYX OF ATROPA BELLADONNA[a]

Sample No.	Temp., ° C.	Wt., ° C.	Alkaloid, %
1	22	25	0.302
2	22	25	0.300
3	22	25	0.299
4	22	25	0.299
5	22	25	0.296
6	22	25	0.295
7	22	25	0.297
		Av.	0.298

TABLE XIV.—RHIZOME AND ROOT OF ATROPA ACUMINATA[a]

Sample No.	Temp., ° C.	Wt., Gm.	Alkaloid, %
1	20	20	0.526
2	20	20	0.532
3	20	20	0.521
4	20	20	0.525
5	20	20	0.526
6	20	20	0.527
		Av.	0.526

[a] All samples were dried by spreading out in a drying room with an average temperature of 22°. Drying time was from four to five weeks.

TABLE XV.—RHIZOME AND ROOT OF ATROPA BELLADONNA[a]

Sample No.	Temp., ° C.	Wt., Gm.	Alkaloid, %
1	20	20	0.505
2	20	20	0.515
3	20	20	0.508
4	20	20	0.510
5	20	20	0.510
6	20	20	0.510
7	20	20	0.510
		Av.	0.510

TABLE XVI.—STEM OF ATROPA ACUMINATA[a]

Sample No.	Temp., ° C.	Wt., Gm.	Alkaloid, %
1	20	10	0.208
2	20	10	0.196
3	20	10	0.201
4	20	10	0.203
5	20	10	0.199
		Av.	0.201

TABLE XVII.—STEM OF ATROPA BELLADONNA[a]

Sample No.	Temp., ° C.	Wt., Gm.	Alkaloid, %
1	20	10	0.195
2	20	10	0.192
3	20	10	0.190
4	20	10	0.195
5	20	10	0.192
		Av.	0.193

[a] The material used as samples in this series of assays consisted of the upper halves of the aerial stems.
Owing to the coarse, splintery nature of the ground drug only 10 Gm. could be placed in the extraction thimble.

TABLE XVIII.—LEAVES OF ATROPA ACUMINATA

Sample No.	Temp., ° C.	Wt., Gm.	Alkaloid, %
1	20	20	0.301
2	20	20	0.332
3	20	20	0.335
4	20	20	0.401
5	20	20	0.335
6	20	20	0.340
		Av.	0.342

TABLE XIX.—LEAVES OF ATROPA BELLADONNA

Sample No.	Temp., ° C.	Wt., Gm.	Alkaloid, %
1	20	20	0.299
2	20	20	0.325
3	20	20	0.324
4	20	20	0.328
5	20	20	0.324
6	20	20	0.328
7	20	20	0.325
8	20	20	0.320
		Av.	0.3216

All samples reported in Tables XIV–XIX were dried by spreading them out in a room with an average temperature of 20°. Drying time was approximately eight weeks.

Various portions of the plant were ground to a moderately coarse powder and extracted in a Soxhlet apparatus. The portion to be extracted was macerated overnight in a menstruum of 8 cc. of stronger ammonia T. S., 10 cc. of ethyl alcohol, and 20 cc. of ether. The following day the extraction was started, ether being added as the extraction menstruum. This extraction was allowed to continue for 24 hours.

The liquid, upon cooling, was transferred to a separatory funnel and the alkaloids were removed from the ether, using approximately half-normal sulfuric acid. Each portion was filtered as it was drawn off. The combined acid portions were rendered alkaline with ammonia T. S. and the alkaloids completely separated by extracting with successive portions of chloroform. The combined chloroform extractions were evaporated to dryness on a water bath and the heating continued for one-half hour. The residue was redissolved in chloroform and again evaporated in the above manner. This procedure was repeated for six successive times. The resulting residue was then dissolved in 4 cc. of chloroform, and 15 cc. of fiftieth-normal sulfuric acid was added. The chloroform was removed by evaporation on a water bath. The solution was allowed to cool, and the excess acid was titrated with fiftieth-normal sodium hydroxide, using methyl red T. S. as the indicator. Results were calculated as hyoscyamine using the U. S. P. XII (24) titration factor.

SUMMARY AND CONCLUSIONS

1. A brief history of the nomenclature is presented with the synonymy and the relevant literature.

2 The leaves, flowers, roots, and fruits of Indian Belladonna are described.

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4. Indian Belladonnda has an average palisade ratio of 8.3 and the European Belladonna an average palisade ratio of 6.1. The vein islet number for the Indian plant is 10.0. There is a variation in the stomatal number per square millimeter of leaf surface. The upper epidermis of the Indian variety showed an average stomatal number of 14.0 and the lower epidermis showed a stomatal number of 93.2. A similar investigation of the European variety yielded the following figures: upper epidermal count, 10.1, lower epidermal count 131.1. The stomatal index for the Indian variety is as follows: upper epidermis 3.4, lower epidermis 17.6. For the European variety: upper epidermis 2.9 and the lower 17.6.

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5. The floral parts of the two varieties are similar in gross structure and insertion but differ mainly in the fact that the Indian variety exhibits larger organs and possesses a corolla which is bright yellow in color.

6. The root and stem histological studies revealed that basically the cellular structures are similar with the exception that the cells of the Indian variety are larger than those of the European.

7. The Indian variety possesses a higher alkaloidal content calculated as hyoscyamine.

8. Drying under infrared lamps at a very high temperature destroys the alkaloidal content.

9. No significant increase of the alkaloidal content was noted following the injection of certain amino acids into the plant tissues of the two varieties.

10. From the past discussion, we conclude that as yet we do not find sufficient variation in the two plants to warrant the present trend of establishing a separate species for the Indian variety. As a result, therefore, we advance the suggestion that, instead of referring to the Indian variety of Belladonna as *Atropa acuminata* Royle ex Lindley, it be called *Atropa Belladonna* L. variety *acuminata*.

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The Identification of Chinese Antimalarial Plants*

By S. T. YANG†

Five definitely identified plants indigenous to China which have been used as antimalarial drugs are briefly described. The present knowledge of these drugs is not complete, but that which is known is reported.

DURING the past five or six years a great deal of interest has been evinced in the study of antimalarial plants both in America and China—in America, because much of the fighting was carried on in the tropics where malaria was a very serious problem, and because, after the fall of Java, the largest source of quinine supply in the world was cut off from the Allies; in China, because the Chinese populace had retreated to the Southwest and established great military, economic, and cultural bastions in Szechuan and Yunnan provinces, the densest malarial region of that country. The southern portion of Yunnan being subtropical in climate and extremely mountainous, malaria, both benign and malignant, rages fiercely in that region. It is an established fact that in times past whole villages and towns were occasionally wiped out by this scourge.

The writer was one of the group of faculty and students connected with the three universities in North China who made the long trek to Kunming and established there the war-time National Southwest Associated University. Being an organic chemist, especially interested in natural and synthetic drugs, his attention was naturally directed to those antimalarial plants indigenous to that region. We also had the good fortune of having several competent botanists on our staff who were devoted students to the native flora. Consequently, in our nine years' stay (eight for the author, because he came to this country in 1945), we became well acquainted with about half a dozen of these plants. It might be of interest to those who are engaged in, or intend to undertake, the study and investigation of antimalarial plants to have available a list of names, describing their occurrence, giving the botanical identification, and summarizing briefly the present knowledge of these substances.

In Chinese books on materia medica, two plants

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are usually classified as antimalarials: *Ch'ang Shan* especially, and *Chai Hu*. For several reasons, their correct botanical identification, however, was not achieved until the last few years. In the first place, malaria is not prevalent in most parts of China, and the people do not have much use for these drugs. In the second place, these plants are found so far in the interior of China that many people have never had an occasion to see them. In the third place, herbs with antifebrile properties are often designated by local druggists as *Ch'ang Shan* or *Ch'ang* substitutes; thus, we have *Tien Ch'ang Shan*, *Tu Ch'ang Shan*, and a host of others. Therefore, a great deal of confusion was encountered in the identification of the original plants designated several thousand years ago by these names. Several plants besides *Ch'ang Shan* were also found to be used for the same purpose by people of different localities. In addition to *Chai Hu*, which has been reported to be *Bupleurum falcatum* Linn., and the two species of *Ch'ang Shan* investigated by the Japanese before World War II, *Orixa japonica* Thunb. (1) and *Hydrangea opuloids* Steud. (2), there are, at present, six plants known to be used in the treatment of malaria. Of the six, five are definitely identified as *Fraxinus malacophylla* Hensl., *Fraxinus chinensis* Roxbg., *Clerodendron yunansis* Hu, *Alstonia yunnansis* Diel, and *Dichroa febrifuga* Lour. It is still not quite settled whether the one found in Szechuan is white or *Pei Ch'ang Shan*.

CHINESE DRUGS

Fraxinus malacophylla **Hensl.**—This plant is found in the southeastern part of Yunnan under the local name of *Pei Chiang Kan*. It grows in the limestone hills of that warm, humid, subtropical region around Wen Shan, Ma Kwan, and Kuang Nan. It is a tree of the ash family and attains a normal height of 20 to 30 feet in its native habitat. A specimen was brought to Kunming several years ago and has been kept alive on the grounds of a local hospital. The bark of the roots, in the form of a dark red powder with a bitter and astringent taste, is used by the local people as an antimalarial; this practice extends back for many centuries. Its use as an antimalarial was first reported by S. K. Liu (3) in 1941. Liu claimed to have isolated an alkaloid which had an antimalarial activity about ten times that of quinine. However, subsequent work by Jang (4) and Tonkin and Work (5) did not substantiate that claim, and no alkaloid was found. The nonalkaloidal constituent had no action at all on *Plasmodium gallinaceum* in chicks. This drug was also tested by the U. S. Government malaria testing station at Memphis, Tenn. (6), against *P. lophurae* in ducks, and the results were also negative.

Fraxinus chinensis **Roxbg.**—This tree is found extensively in Szechuan and Yunnan, and, in fact, is one of the group used in the production of Chinese white wax or insect wax. It thrives best in moist places, along river banks or at the edge of a lake; hence, it is called *Sui* (water) *Pei La Shu* (white wax tree). It grows to a height of 4 or 5 to 10 or 12 feet. Originally, this plant was not used as an antimalarial by the Chinese people. As mentioned before, the tremendous migration of the Chinese people from the coastal provinces into the southwest interior, during our long war with Japan, brought arsenals and factories to Szechuan and Yunnan. The authorities were immediately confronted with the problem of malaria, and, because of the shortage of quinine and other modern antimalarial drugs, a fanatic search of native cures was started in 1941 and 1942. A Czechoslovakian medical doctor by the name of Schroeder, on the staff of the Applied Chemical Institute of the Chinese Ordnance Administration at Luchow, Szechuan, took note of this tree, because it was of the same family as the European ash, *Fraxinus excelsior* Linn. He told his colleagues that the bark of the European ash was used as an antimalarial by the people of the Balkans during the first World War, and it was probable that the bark of this Chinese tree might have the same property. Pressed by actual needs, and hearing that in Yunnan another tree of the same family was being used by the people there as an antimalarial, the institute authorities immediately began a study of this plant Since the bark of *Fraxinus excelsior* had been investigated by Austrian chemists, who isolated a glucoside, Fraxin, and its aglucone, Fraxetin (7)—although no mention was made of its use as an antimalarial—Dr. K. H. Lih, the chemist-in-charge, and his colleagues extracted Fraxetin from the bark of the Chinese wax tree in 1942 (8). They made pills containing 10 per cent Fraxetin and conducted clinical experiments with malaria patients in the arsenals at Luchow. After two years they claimed that "Fenodin"—the name given to this tablet—was quite effective against both *P. vivax* and *P. falciparum*. Samples were sent to this country and Great Britain by Dr. Joseph Needham, of Cambridge University, England, who organized the Sino-British Science Coöperation Office in Chungking during the latter stages of the war. The tests on *P. lophurae* in ducks and *P. gallinaceum* in chicks were negative Jang's results (4) also indicated that it was ineffective clinically, in the small number of cases tested.

Clerodendron yunansis **Hu.**—This is a tree or tall shrub common in the environs of Kunming. It is very ornamental due to large masses of fine white flowers. Most Chinese books on materia medica describe two different kinds of *Ch'ang Shan:* one is *Yichow Ch'ang Shan* which has now been identified as *Dichroa febrifuga*, and the other, *Haichow Ch'ang Shan*, as *Clerodendron trichotomum*. Haichow was the old name for Kiangsu and Anhui. This Yunnan species is therefore frequently called *Tien Ch'ang Shan*. The majority of the drugstores in Kunming use thin slices of the twigs of this tree for prescriptions calling for *Ch'ang Shan*. On account of an unpleasant odor from its crushed leaves, it is also called by the local people *Hsiu* or foetid, *moulan shu*. So far as is known, no investigation has been conducted with this plant.

Alstonia yunansis **Diel.**—This is a small shrub about 2 to 3 feet in height and grows wild in the fields of Kunming. Occasionally, it is planted in

gardens. Its profusion of small, bright red flowers and slender pea-like seed-pods is very picturesque. It is called *Chih Ku* or chicken bone *Ch'ang Shan* in Kunming (also in the book, "Identification of Chinese Plants, Drawings and Descriptions," Commercial Press, Shanghai), although this name is also used elsewhere to designate the best grade of *Dichroa* roots. The bark, twigs, and seed-pods are all used as the drug by an old and famous drug house in Kunming, Fu Ling Tang. It contains several alkaloids and resinous substances which show color changes in acid solution. It was found inactive against *P. lophurae* in ducks by the Memphis station (9).

Dichroa febrifuga Lour.—This is an evergreen shrub found in the high tablelands of Szechuan and Yunnan. The main centers in Szechuan are Chungking, Hochuan, Ching-Fu Shan, and Wan Hsien; and in Yunnan, they are Yungping, Lungkai, Ch'angkai, and Tali. *Dichroa febrifuga* grows to a height varying from 4 or 5 to 9 feet. The leaves are opposite and lanceolate; and the flowers, on a pyramidal panicle, are numerous, small, and violet to blue in color, when fresh, and black when dry. The ripe fruit, about the size of a seed corn, contains many small seeds covered with fuzz. In Szechuan, only the roots are used; they are colored bright yellow. The use of the roots is strictly in accordance with the recommendations of Chinese books on materia medica. In fact, the name *Ch'ang Shan* is applied to roots only. The effectiveness of the leaves and young shoots was also noted in those classical books, but it was said *Shu Chi*, the name for the shoots and leaves, causes a stronger irritation to the stomach. In Yunnan, however, the dried leaves and sometimes the twigs are used, but never the roots. So, for quite a while we did not associate the Szechuan and Yunnan products to the same plant, knowing the loose use of the name *Ch'ang Shan* by local druggists.

This is the only antimalarial plant in this entire group, the effectiveness of which against avian and human malarias has been established beyond any doubt (3–6, 10). The leaves are found to be far superior to the roots in their effectiveness as an antimalarial.

It was not until the latter part of 1941 that we were able to obtain a small quantity of the powdered leaves. This drug was in the hands of a powerful local firm which wished to keep its source a trade secret from the public, especially the immigrants from outside. This company usually bought up most of the supply at its source, boiled the leaves with water into a thick black syrup, and then shipped the same to their Kunming factory for the manufacture of pills, under the trade name of "Chunine," and other galenical forms. Therefore, it was difficult to get any leaves as such in Kunming. Our first supply, from a friend, was quite small in quantity and finely powdered, so that we could not distinguish the original shape of the leaves. We learned, however, where the plants could be found. We had planned to send out a party to collect it in its native habitat, Yungping, in sufficient quantity for chemical studies, when, unfortunately, the Japanese Army invaded Burma in the beginning of 1942 and soon pushed into Yunnan. That portion of Yunnan on the western bank of the Salween River fell into enemy hands, and the region between Tali and the Salween River, where this plant is found, became the battle front. It was therefore impossible to send any party into that region for two years. The Japanese were finally mopped up in that section in the spring of 1944. That summer, with the help of the Chinese Army, an assistant was finally sent. After a difficult and hazardous journey, he visited all the three locales, but succeeded in procuring only a few pounds of the dried leaves and another few pounds of the twigs. He did bring back, however, two live specimens of the plant in question as well as some pressed specimens of the flowers and fruit. The reason for his meager success in securing a large amount of the leaves was that he had gone at the wrong time! The local drugstores usually keep about 25 to 30 pounds of the drug on hand, and seldom allow anybody to have more than a few ounces at a time, especially in view of the long malaria season with no fresh supply available until the next spring. Large quantities of the drug can only be had during the annual fair at Tali in the earlier part of April, or, more correctly, the middle of the third month according to the old Chinese calendar. The fair at Tali is quite a large affair; it is not only attended by merchants throughout Yunnan province but also by those from Szechuan, Hsi-Kang, Chinghai, Tibet, Burma, and India. Several thousand pounds of the leaves of *Dichroa febrifuga* can be purchased at that time. Thus, it was necessary to send another man the following spring to secure a sizable quantity. In the meantime, of course, we had identified the plant to be *Dichroa febrifuga*, identical with the Szechuan plant. The late Professor Y. T. Wu and Mr. Cho Po Chien, of Tsing Hua University, identified these different plants.

After identification, the most pressing problem was the isolation of the active principle or principles in pure chemical form. Jang in 1944 (4) claimed to have isolated two alkaloids from the root bark, but the work of several other investigators, including the writer (5, 6), has not confirmed his results. Some of us working on the root bark and others on the leaves have not been able to isolate any alkaloid thus far.

SUMMARY

There are six medicinal plants in China which may have antimalarial action; namely, *Fraxinus malacophylla* Hensl., *Fraxinus chinensis* Roxbg., *Clerodendron yunansis* Hu, *Alstonia yunansis* Diel, *Dichroa febrifuga* Lour., and an unidentified species.

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The Pharmacologic and Chemotherapeutic Action of *p,p'*-Diaminodiphenyltrichloroethane*

By M. I. SMITH, J. M. JUNGE, and W. T. McCLOSKY†

The toxicity, absorption, retention, metabolic fate, and chemotherapeutic action of p,p',-diaminodiphenyltrichloroethane (DADT) were studied in comparison with the two analogs p,p',-dichlorodiphenyltrichloroethane (DDT) and p,p', - diaminodiphenylsulfone (DDS), and the results are reported.

In a previous study (1) of the pharmacologic action of a series of compounds chemically related to the insecticide DDT (*p,p'*-dichlorodiphenyltrichloroethane) it was shown that the characteristic neurotoxic and hepatotoxic actions of this substance are dependent on five halogens in the molecule. Compounds without the aromatic chlorine and compounds with one or more of the aliphatic chlorines removed had little or none of the actions of DDT on the central nervous system or the liver. Evidence of renal excretion of DDT and the chemically related chlorinated compounds or their degradation products was obtained by the demonstration of the presence of organic chlorine in the urine of rabbits treated with these compounds. Moreover, in the case of DDT it was shown that the excretory product in the urine of rabbits was the partially dechlorinated derivative *p,p'*-dichlorodiphenyl acetic acid (2, 3).

The present report concerns the pharmacologic properties and metabolic fate of another compound chemically related to DDT, a diamino analog of DDT, to be referred to for brevity as DADT. This compound, which was synthesized at the suggestion of one of us (M. I. S.) in the laboratories of the Geigy Chemical Co. and kindly supplied by Dr. V. Froelicher, seemed of particular interest for two reasons. First, it can be diazotized by the method of Bratton and Marshall (4) and therefore can be traced with relative ease and accuracy in the body tissues and fluids; secondly, it appeared to offer potential possibilities of chemotherapeutic activity because of its chemical relationship to *p,p'*-diaminodiphenylsulfone (DDS), the parent substance of many derivatives studied in this and in other laboratories for chemotherapeutic activity in experimental tuberculosis with a measure of success (5–8).

* Received April 9, 1948, from Experimental Biology and Medicine Institute, National Institute of Health, Bethesda, Md.

† Deceased January 12, 1948.

The structural relationship of this compound to DDT and DDS is shown below.

DDT $Cl—C_6H_4—\underset{\underset{C(Cl)_3}{|}}{\overset{\overset{H}{|}}{C}}—C_6H_4—Cl$

DADT $NH_2—C_6H_4—\underset{\underset{C(Cl)_3}{|}}{\overset{\overset{H}{|}}{C}}—C_6H_4—NH_2$

DDS $NH_2—C_6H_4—SO_2—C_6H_4—NH_2$

EXPERIMENTAL

The *acute toxicity* of DADT was studied in rats and mice. The base, being insoluble in water, was administered orally as an aqueous suspension with gum acacia or as a solution in oil.[1] It can also be administered orally or subcutaneously as the hydrochloride in 10% aqueous solution at a *p*H of 2.5–3.0, the substance being dissolved in a minimum volume of dilute HCl and made up to volume with H_2O. The results indicate that it appears to be absorbed best from oil-acetone solutions on oral administration.

Table I shows the results of the experiments on the acute toxicity of DADT and DDS in rats. The approximate LD_{50} of DADT in rats is between 1.0 and 1.5 Gm./Kg., and is therefore a little more than one-tenth as toxic as DDT, which is only 0.15 Gm./Kg. (9), thus following the general pattern of other DDT related compounds in which the aromatic chlorines are either lacking or replaced by other substituents (1). It is also somewhat less toxic than DDS in the rat.

Table I.—Acute Toxicity in Rats. Oral Administration

Dose, Gm./Kg.	No. of Rats Used	No. Died	Per Cent Mortality
DADT 10% in corn oil			
2.0	3	3	100
1.5	10	8	80
1.0	20	4	20
DDS 10% aqueous suspension with gum acacia			
1.0	10	6	60
0.8	10	3	30

The systemic effects of DADT differ from those of DDT or DDS. Following the oral administration of DADT no symptoms are noticeable for an hour or two. Following this there is dyspnea, gen-

[1] It is rather sparingly soluble in oil, but readily soluble in acetone. A 10% solution of the substance in corn oil can be made conveniently by dissolving the substance in a minimum volume of acetone, 1 Gm. per cc., then adding the requisite amount of oil to volume.

eral muscular weakness, incoordination, ataxic gait, progressively difficult and labored respiration, coma, and death. Death may occur with large doses as 1.5 to 2.0 Gm./Kg. in two to five hours, or with smaller doses, as 1.0 Gm./Kg., in one to three days. There is no hyperirritability as in DDS poisoning, nor tremors and convulsions as in DDT poisoning.

The acute toxicity of DADT in mice is higher, as shown in Table II, than DDT, and somewhat lower than DDS. There is no adequate explanation at this time for the differences in susceptibility of the rat and mouse to these compounds.

TABLE II.—COMPARATIVE ACUTE TOXICITY IN MICE

Compound	Administration	Gm. per Kg.	No. Used	Per Cent Mortality
DDT	Oral in peanut oil	0.8	10	100
		0.6	10	100
		0.4	10	40
DADT	Oral in corn oil	1.0	10	100
		0.5	10	90
		0.25	10	40
DDS	Oral in gum acacia	0.5	10	60
		0.25	10	70
		0.10	10	0

linear curve, shown in Fig. 1, obtained by plotting absorption against concentration.

Blood levels in rats and guinea pigs, from single doses of 1.0 and 0.5 Gm./Kg., respectively, were low, of the order of 0.5 to 1.5 mg. per cent throughout the twenty-four-hour period following the administration of this compound. This probably is not due to poor absorbability since rats receiving 1.0 Gm./Kg. usually showed definite symptoms of intoxication, sometimes persisting for twenty-four to forty-eight hours. More likely it is a matter of distribution of the substance in the body favoring certain tissues in preference to the blood, but this has not been determined definitely.

In rabbits the blood levels varied in different animals (Table III). Usually blood levels of 2 to 8 mg per cent were attained within a few hours following a single dose and this was maintained for several days. The excretion in the urine is greatest during the first and second days, but it continues for many days thereafter. Measurable amounts can be detected in the urine for ten to twenty days. The persistence of the substance in the urine for many days is strikingly analogous to DDT, for as has been shown previously organic chlorine, signifying DDT or degradation products, may persist in the urine of rabbits for ten days or longer following a single dose of DDT (10). On the other hand, the blood levels of DADT in rabbits resemble more nearly those of DDS (6), the blood levels of DDT in rabbits being usually of a very low order (10). Thus it would seem that DADT stands as an intermediary between DDT and DDS, having the characteristics of the former in so far as persistence of action is concerned, with absorption and distribution qualities of the latter.

TABLE III.—BLOOD LEVELS AND URINARY EXCRETION OF DADT IN FOUR RABBITS, WEIGHING 2–2.5 KG ORAL ADMINISTRATION OF 0.5 GM./KG., 10% SOLUTION IN CORN OIL

Time	Blood Levels, Mg. % 1	2	3	4	Urinary Excretion, Mg. Total 1	2	3	4
2–4 Hr.	7.0	2.0	2.2	6.1	..	...	...	...
24 Hr.	8.5	2.0	6.0	8.0	92	156	163	144
48 Hr.	...	0.8	4.5	4.2	12	50	69	196
72 Hr.	...	1.0	3.7	1.0	12	12	11	68
96 Hr.	3.0	0.4	1.0	...	12	2	12	22
6 days	1.0	...	...	...	5	9	2	11
7–8 days	0.8	0.4	0.3	0	6	10	...	...

The acute toxicity in other animal species has not been studied thoroughly, but twelve guinea pigs and eight rabbits given single doses of 0.5 Gm./Kg. in corn oil by stomach tube for metabolic studies survived and showed no visible toxic effects. If it is remembered that rabbits almost invariably show definite tremors from 0.05 Gm./Kg. DDT (9) and that the LD_{50} of DDT for rats is 0.15 Gm./Kg., while guinea pigs usually succumb to 0.25 Gm./Kg. DDS (6), it would appear that DADT is less toxic than the other two compounds in both species.

Metabolism.—The metabolism of DADT was studied in rats, guinea pigs, and most thoroughly in rabbits. The concentration of the substance in the blood and urine was studied by a modified Bratton and Marshall (4) technique. Ten to fifteen minutes are required for the complete formation of chromogen on diazotization and coupling and during this time much of the chromogen forms colloidal aggregates, as in the case with many sulfones. For this reason 5 cc. of acetone was added in all cases just before reading to redissolve the chromogen. The Fisher Electrophotometer was used with B 525 filter, and the light absorption recorded. From these data the concentration of DADT was computed from the standard

In our studies on the metabolic fate of DADT advantage was taken of its physicochemical characteristics, *viz.*, the insolubility of the base in water and its solubility in organic solvents, and the solubility of the hydrochloride in water and its insolubility in ether. It was noted that the urine of rabbits, which is normally alkaline, was strongly opalescent, almost milky in appearance for two to three days following the administration of a single dose of DADT. This completely cleared when shaken with ether or when strongly acidified with HCl. The following procedure was therefore adopted for the isolation of the substance from the urine.

The urine was alkalinized with dilute NaOH solution to *p*H 10–11, shaken out two or three times with ether, the ether and emulsion layer if any dehydrated with anhydrous Na_2SO_4, and washed once or twice with water. The washed ether was then extracted thoroughly by shaking in a separatory funnel two or three times with dilute HCl in H_2O

at *p*H 2.0 or less. The acid aqueous extract was then alkalinized with NaOH to complete precipitation and this extracted with two or three portions of ether. This procedure can be repeated several times without appreciable loss of material until the acid aqueous extract is almost colorless. The last traces of pigment were removed by shaking with a small amount of charcoal (Nuchar W) and filtered. The colorless filtrate was then alkalinized with sufficient NaOH solution to complete precipitation, the precipitate centrifuged off and washed with water by centrifugation. The packed precipitate was dissolved in a few cc. of hot ethyl alcohol in which it is very soluble. The alcoholic solution was allowed to cool, and any insoluble material was removed after centrifugation. To the decanted clear alcoholic solution 2 volumes of water was added cautiously until slight turbidity began to appear. On standing for several hours, with frequent rubbing of the sides of the centrifuge tube with a glass rod, crystallization occurred and this was allowed to proceed until the supernatant liquid was clear and remained clear on further addition of small amounts of water. When crystallization was complete the material was centrifuged off, the supernatant liquid decanted, and the centrifugate dried *in vacuo*.

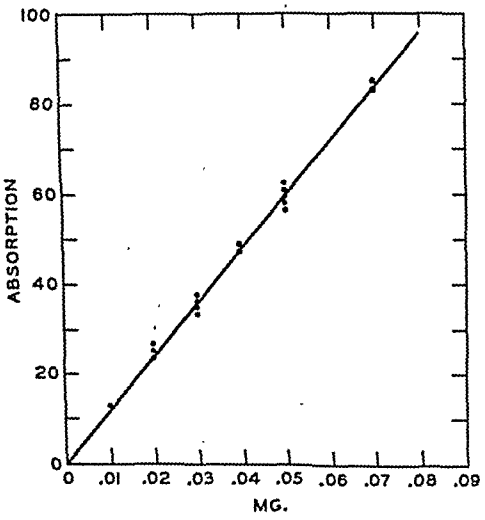

Fig. 1.—Absorption-concentration curve of DADT as measured by the Fisher Electrophotometer with B 525 filter. Ordinates show light absorption; abscissae-concentration in mg. per 18 cc., including 5 cc. acetone.

Good yields were thus obtained, in relation to the material originally present in the urine based on determinations by the diazotization technique. This crystalline material had a sharp melting point of 146–8°.[2] A solution of this gave upon diazotization and coupling an absorption-concentration curve quantitatively identical with that of the compound itself. Moreover, DADT and the substance isolated from the urine, when nitrated in equivalent amounts and a benzol solution of the nitrated compounds treated with sodium methylate in methanol by the method of Schechter and Haller (11), gave identical absorption curves within the range of 460–700 mμ, with a maximum absorption in both cases at 580–600 mμ, as shown in Fig. 2.

Microanalyses,[3] of a sample of DADT and two samples isolated from the urine of rabbits gave the following results.

	Carbon	Hydrogen	Chlorine
DADT	53.41	4.22	32.54
Urine sample one	53.73	4.73	32.60
Urine sample two	53.54	4.66	32.04
Theory	53.27	4.15	33.10

These results indicate that the material extracted from the alkalinized rabbit urine is probably unaltered DADT. It is probable that DDS is excreted in a similar way, though accurate information on this is not available. The compound DDT, on the other hand, it has been abundantly demonstrated (2, 3), is excreted in the urine of rabbits as *p,p'*-dichlorodiphenyl acetic acid. In this respect, therefore, DADT *resembles DDS more closely than DDT.*

Chemotherapeutic Activity.—The chemotherapeutic activity of DADT was examined in experimental pneumococcus infection (Type I) in mice, and in experimental tuberculosis in guinea pigs. The antipneumococcal tests in mice were carried out in the usual manner (12). The mice were inoculated intraperitoneally with 0.5 cc. of 10^{-6} dilution of a six-hour veal infusion blood broth culture, which kills all controls in twenty-

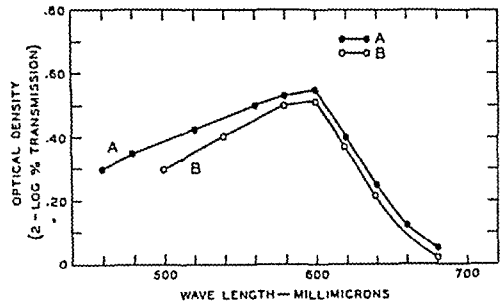

Fig. 2.—Absorption curves of nitrated DADT in 5 cc. benzol + 10 cc. of 10% sodium methylate in methanol. Bausch & Lomb universal spectrophotometer. All measurements made at a depth of 1.5 cm.

A. 2 mg. DADT. *B*. 1.8 mg. of material isolated from rabbits' urine.

four to forty-eight hours, and the drug was administered for four days twice daily in doses of 20 to 50 mg./Kg. The individual doses were from 8 to 20% of the LD_{50}. For comparison groups of infected mice were also treated with equivalent doses of DDT and 75 mg./Kg. of DDS. The results shown in Table IV indicate no chemotherapeutic activity from DADT or DDT, with a fair degree of chemotherapeutic activity from DDS, as has usually been seen in this laboratory in routine testing of sulfone derivatives, with DDS being used as a standard of reference (12).

In the test for chemotherapeutic activity in experimental tuberculosis twenty male guinea pigs of

[2] The melting point of the DADT used was 153–4°. Mixed melting point 148–9.

[3] For this we are indebted to C. A. Kinser of this Institute.

about 300 Gm., each were inoculated intraperitoneally with 0.5 mg. tubercle bacilli human strain suspended in 1 cc. sterile salt solution. One group of 10 was kept as controls and the animals of the second group were treated daily with 0.25 Gm./Kg. DADT for the first two weeks, and 0.10 Gm./Kg. for the following eight weeks. The dose was reduced on account of evidence of toxicity, probably due to cumulative action. All but two of the controls died in forty-seven to ninety days, and all of the treated animals died in seventeen to ninety days. The two surviving controls were then killed and the experiment terminated. At autopsy the extent of tuberculous involvement was noted and rated as previously described (6). The T. B. index of the ten controls varied from 4 to 16 with an average of 10.7 out of a possible maximum of 20. In the group of treated animals the range was 7 to 18 with an average of 12.2. In a group of 10 animals similarly infected and treated concurrently with 10 mg./Kg. streptomycin twice daily intramuscularly all survived and when killed and autopsied the extent of tuberculous involvement ranged from 1 to 4 with an average T. B. index of 1.5. Obviously DADT had no therapeutic effect in tuberculosis under the experimental conditions.

TABLE IV.—THERAPEUTIC TEST. PNEUMOCOCCUS (TYPE I) INFECTION IN MICE

Drug	Total Dose, Gm./Kg. per os	No. of Mice	No. Died	Survival, %
DADT	0.160	15	15	0
	0.400	15	14	6.6
DDT	0.160	15	15	0
	0.400	15	15	0
DDS	0.600	14	8	42.8

DISCUSSION

As this work was well under way and nearing completion a report appeared by Burger and associates (13) on the preparation of a dibenzoyl derivative of DADT, 1,1,1-trichloro-2,2-bis-(*p*-benzamidophenyl)-ethane, which is stated to have had a high inhibiting action *in vitro* against the tubercle bacillus. They were apparently unable to get the free base. Shortly afterward Kirkwood and associates (14) reported on the synthesis of DADT with a melting point of 92–95°, which inhibited the growth of the tubercle bacillus *in vitro* in a concentration of 1:100,000 and *in vivo* tests "showed remarkable control of experimentally induced tuberculosis in guinea pigs." In a later publication Kirkwood and Phillips (15) reported the preparation of DADT with a melting point of 154–55° with a tuberculostatic activity *in vitro* of 1:100,000 and with indications of possible activity *in vivo*, but with no details. Our compound had a melting point of 153–54°. It was so insoluble in the usual liquid media at *p*H 7.0 that *in vitro* tests for tuberculostatic activity were not considered feasible.

It may be of interest to comment briefly on the relation of chemical constitution to pharmacologic and chemotherapeutic action as exemplified by the three compounds in this study. The toxicity of DADT in some species as the rat and rabbit is less than that of DDT and is consistent with previous findings which indicated that dechlorination of the DDT molecule resulted in compounds of lower toxicity. The diamino substituted derivative is also free of neurotoxic action so characteristic of DDT. The substitution of two amino groups in place of the two aromatic chlorines in DDT has had the effect of giving much higher blood levels, and of blocking the metabolic degradation of the trichloroethane bridge to the acetic acid derivative. Comparing DADT with DDS it is evident that the trichloroethane bridge makes for lower toxicity and much longer retention in the body, while all chemotherapeutic activity possessed by the sulfone appears to have been completely eliminated by the substitution of the trichloroethane group for the SO_2 group.

SUMMARY AND CONCLUSIONS

The toxicity, absorption, retention, metabolic fate, and chemotherapeutic action of *p,p'*-diaminodiphenyltrichloroethane (DADT) were studied in comparison with the two analogs *p,p'*-dichlorodiphenyltrichloroethane (DDT) and *p,p'*-diaminodiphenylsulfone (DDS).

DADT is usually less toxic than DDT and is devoid of the neurotoxic actions of DDT. It is fairly well absorbed from the gastrointestinal tract, giving blood levels comparable with those of DDS, and is retained in the body for many days, like DDT. That part which is excreted in the urine appears to be unchanged and, unlike DDT, it is not metabolized to an acetic acid derivative.

DADT has no chemotherapeutic activity in experimental pneumococcus infection in mice or in experimental tuberculosis in guinea pigs.

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An Investigation of the Assay of Ascaridol. II. The Iodination of Terpenes*

By ALFRED HALPERN†

The rate of iodine released in the assay of ascaridol was determined. The major portion of the iodine was released within the first minute of the reaction. A slower steady release of iodine was also evidenced. The possibility of iodinating unsaturated hydrocarbons structurally related to ascaridol was investigated. The olefinic linkage in these compounds was not iodinated under the conditions of the official assay for ascaridol. The nonstoichiometric behavior of ascaridol toward the iodide ion cannot be explained by the simple iodination of olefins.

In an earlier investigation of the assay of ascaridol in oil of chenopodium, some of the difficulties involved in the determination were reported (1). The method that had the fewest limitations was the procedure of Cocking and Hymas (2) involving the liberation of iodine from acidified potassium iodide by the organic peroxide ascaridol. This method is official in N. F. VIII.

One of the chief criticisms of this method was the use of an empirical equivalent in the calculation of the ascaridol content. The reaction between the ascaridol and the potassium iodide does not proceed in the expected stoichiometric relationship, but one mole of iodine is equivalent to 0.66 mole of ascaridol. This was determined from the mean of a series of experiments. It is important to note that this relationship was reproducible. The authors (2) claimed that three reactions occurred in sequence: the liberation of 2 moles of iodine from the acidified potassium iodide solution, followed by an unexplained liberation of iodine, and then when the solution was diluted, an absorption of iodine took place.

The iodometric determination of peroxides is a rather well-known procedure (3–5). Most of these determinations are similar to the one proposed by Cocking and Hymas. The estimation of peroxides by these techniques was quantitative for many peroxides. However, difficulties were noted in the case of others (e.g., cyclohexene-peroxide).

In the ascaridol assay the absorption of iodine might be explained by the addition of iodine to an unsaturated olefin. Ascaridol has an unsaturated linkage in its molecule. The diiodide, if formed, might also be reduced by the hydrogen iodide present, to liberate free iodine, thus accounting for the nonstoichiometric quantity of iodine present. Another explanation would be the reaction between the decomposition products of ascaridol and hydrogen iodide to liberate free iodine.

The official determination of ascaridol content is carried out in the presence of the iodide ion. Iodine, in the presence of the iodide ion, forms the triiodide ion. The triiodide ion has been shown to inhibit the iodination across the unsaturated linkage (6). The presence of ascaridol, a peroxide, might catalyze this addition.

The present study was primarily concerned with the behavior of unsaturated olefins related to ascaridol under the experimental conditions of the assay. The possibility of iodinating these compounds and the influence of these iodides, if formed, on the quantity of iodine liberated during the course of the reaction was investigated.

EXPERIMENTAL

Oil of Chenopodium (d_{25} = 0.959; n_D^{25} 1.4768) was assayed for ascaridol content by the iodine method (N. F. VIII) and the average of five determinations corresponded to 69% ± 4% of ascaridol. In order to determine the rate of iodine released, the assay was repeated on another sample of the same oil. After the indicated lapse of time a 1-ml. aliquot was withdrawn and the iodine content immediately determined by titration with hundredth-normal sodium thiosulfate solution. The amount of iodine liberated and the equivalent ascaridol content were determined (Table I and Fig. 1).

TABLE I.—IODINE LIBERATION DURING THE ASSAY OF ASCARIDOL

Time, Min.	Iodine Mg.[a]	Ascaridol Equivalent, %[b]	
1	13.1	59.8	
2	14.1	62.1	
3	15.5	66.3	
4	15.6	66.4	End of N. F. VIII time period
5	16.0	69.8	
7	16.6	73.2	
10	16.9	74.5	
15	17.0	74.8	
20	17.1	75.1	

[a] Present in a 1-ml. aliquot. The indicated quantities represent the mean of three determinations.
[b] Based on a 0.2750-Gm. sample in 23 cc. of reaction medium.

The results of the iodine-release study indicated that the greatest amount of iodine was liberated

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within the first minute of the reaction period. There was a steady release of iodine throughout the entire period of observation (twenty minutes). Between zero and one minute 13.1 mg. of iodine was liberated, corresponding to 59.8% of ascaridol (based on a 0.275-Gm. sample) and during the nineteen-minute period following an additional 4 mg. of iodine was liberated, corresponding to 15.3% of ascaridol. There was no observable absorption of iodine during this period. This was in agreement with the observation of Cocking and Hymas (2) that there was an immediate liberation of iodine followed by a second unexplained liberation of iodine.

The cyclic peroxides of the 1,4 type and their conjugated polymers have been shown to behave differently toward the ferrous and iodide ions (6, 7). The reaction proceeds much more slowly with the bridge-type peroxides than with the simple dihydroperoxides. Ascaridol, a monomeric 1,4 bridge-type cyclic peroxide, might be expected to react at a slower rate and the results in this case would be empirical. The rate study, however, implies that two reactions take place and that the first reaction proceeds at a much greater rate than the second one. We hesitate to draw conclusions from a limited amount of data and a more intensive study of the kinetics of the reaction is now in progress. These results will be published at a later date.

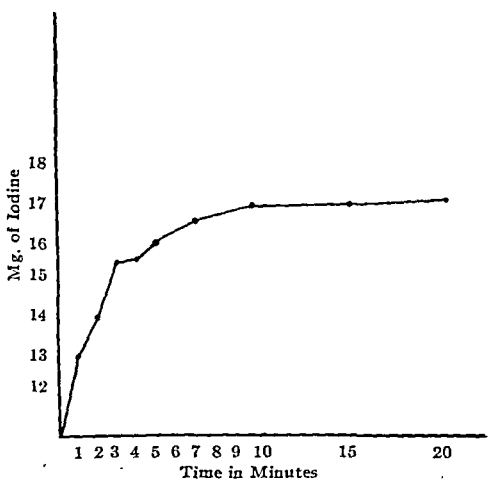

Fig. 1.—Iodine Liberation During the Assay of Ascaridol.

Ascaridol has been considered structurally and chemically similar to the auto-oxidation product of 1,3 menthadiene (7). Presumably when the oxygen is given up an olefinic compound results. This would be expected from analogy to related compounds, such as rubrene peroxide which forms rubrene on decomposition (8). The formation of olefins might account for the absorption of iodine reported by Cocking and Hymas (2). To investigate adequately the role of the unsaturated compounds in the possible absorption and latent liberation of iodine, a series of olefins related to ascaridol were studied in the light of their tendency to be iodinated under the condition of the assay.

Preparation of *p*-Menthene.—*p*-Menthene was prepared by the dehydration of menthol with copper sulfate in a manner similar to the one described by Richtman and Kremers (9). Presumably, the resultant menthene was a mixture of Δ^2-menthene and Δ^3-menthene. Since it was not important for the purposes of this investigation to obtain the pure individual isomer the mixture was distilled and the fraction boiling at 171–174°/760 mm. was collected. It had the following physical properties: d_{20} = 0.826, n_D^{20} = 1.4601.

The nitrosochloride was prepared in the usual manner and melted at 90–91°.

Preparation of Terpinolene.—In a 1-liter, 3-neck flask fitted with a dropping funnel and an inlet tube for steam and an exit tube connected to a condenser, was placed 500 ml. of a saturated oxalic acid solution This solution was heated to boiling and a mixture of 30.8 Gm. (0.2 mole) of terpineol in 100 ml. of hot (80–90°) water was added dropwise. A current of steam was passed through the reaction medium to remove the terpinolene as soon as it was formed. When all of the terpineol was added, the steam distillation was continued for another fifteen minutes. The distillate was extracted with three 50-ml. portions of petroleum ether and dried over anhydrous sodium sulfate. The solvent was removed under diminished pressure and the residue distilled. The fraction boiling at 67–68° at 10 mm. of mercury pressure was collected. The micro boiling point at atmospheric pressure was 184–185°. Yield: 56% of theory. The tetrabromide was prepared and it melted at 116–117°.

d-Limonene, dipentene and *p*-cymene were obtained commercially and redistilled. The fraction boiling over the described range (Table II) was collected and the physical properties determined.

IODINATION STUDY

I. Iodination Study

Approximately 1 mM of the appropriate unsaturated compound, accurately weighed, in 5 cc. of 90% acetic acid, was added to a 125-ml. Erlenmeyer flask containing a solution of 5 mM. of iodine in 5 ml. of hydrochloric acid and 10 ml. of glacial acetic acid, previously cooled to −3°. The flask was stoppered and allowed to stand in a cool place for ten minutes. The temperature was maintained at 0–5°. The contents were then titrated with 0.1 *N* sodium thiosulfate solution. The amount of iodine absorbed by the experimental olefins was determined (Table III).

II. Iodination of the Presence of the Iodide Ion and the Triiodide Ion

This procedure was the same as described above (I) except that 3 cc. of an 83% (*w/v*) potassium iodide solution was added to the iodine-HCl-acetic acid mixture prior to the addition of the unsaturated compound. The iodine content was determined at the end of ten minutes by titration with 0.1 *N* sodium thiosulfate solution (Table III).

The unsaturated compounds all absorbed appreciable quantities of iodine, with the exception of *p*-cymene which was a benzene derivative and consequently would not be expected to iodinate readily under the conditions of the experiment. Iodination in the presence of the iodide ion, however, resulted in

an almost quantitative recovery of iodine, indicating no iodination of the olefinic linkage. The triiodide ion has been shown to depress the iodination of unsaturated compounds. Wagner, Smith, and Peters (6) have studied the iodination of cyclohexene and isoprene in the presence of the triiodide ion, as a phase of an investigation of the determination of peroxides and have noted similar results.

SUMMARY

1. The rate of iodine released in the assay of ascaridol was determined. The major portion of the iodine was released within the first minute of the reaction. A slower steady release of iodine was also evidenced.

TABLE II.—PHYSICAL PROPERTIES OF TERPENES

Compound	760 Mm.	Specific Gravity, d^{24}	Refractive Index, n_D^{20}	Source
Menthene	171–174°	0.826[a]	1.4601	Synthetic from menthol
d-Limonene	174–176°	0.849	1.4749	Eastman Kodak
Dipentene	175–176°	....	....	Eastman Kodak
Terpinolene	184–185°	0.854	1.4840	Synthetic from terpineol
p-Cymene	176.5–177.5°	0.860	....	Eastman Kodak

[a] d^{20}.

TABLE III.—THE IODINATION OF TERPENES

Compound	Millimoles Present	Method I: Millimoles of I_2 Added[a]	Method I: Millimoles of I_2 Found	Method II: Millimoles of Iodine Present as I_3^-[b]	Method II: Millimoles of I_2 Found
Menthene	1.12	5.0	4.1	5.0	4.98
Terpinolene	1.41	5.0	2.8	5.0	4.99
d-Limonene	1.61	5.0	2.6	5.0	4.99
Dipentene	1.53	5.0	2.9	5.0	4.98
p-Cymene	1.43	5.0	4.5	5.0	5.00

[a] In 5 cc. of glacial acetic acid.
[b] Iodine added to the KI solution so that 3 cc. of solution contains 5 mM of I_2.

To establish the possible effect that peroxides might have on the iodination of the unsaturated compound, the reaction was repeated in the manner described for Method II and 0.01 mole of ascaridol was added with the olefin. A blank was run at the same time without the olefin. The iodine liberation due to the ascaridol was determined and subtracted from the experimental value. The results indicated that there was no absorption of iodine and were identical with Method II. This conclusively established that the olefins were not iodinated under the conditions of the experiment.

Since it was established that the iodination of the olefinic linkage did not occur under the conditions of the assay, the possibility was nonexistent for the iodides to become a source for the unexplained secondary liberation of iodine. For this reason the experimental determination of the stability of these iodides in the presence of hydrogen iodide and ascaridol was not carried out at this time. However, it was of interest to note that a simple alkyl iodide (ethyl iodide) was reported (6) to be perfectly stable under conditions analogous to those of the N. F. VIII for the assay of ascaridol.

It was evident from the above results that an explanation for the atypical behavior of ascaridol toward the iodide reagent was not the simple iodination of the olefinic linkage.

2. The possibility of iodinating unsaturated hydrocarbons structurally related to ascaridol was investigated. The preparation of menthene and terpinolene was described.

3. The olefinic linkage in these compounds was not iodinated in the presence of the triiodide ion.

4. The nonstoichiometric behavior of ascaridol toward the iodide ion cannot be explained by the simple iodination of olefins.

5. Further work is now in progress on an investigation of the kinetics of the reaction and the intermediates formed in the course of the determination.

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A Study of the U. S. P. XIII Method for the Assay of Desiccated Thyroid*

By HAROLD C. HEIM

A reagent blank titration and a "slipping" end point are frequently encountered in the U. S. P. XIII method for the determination of iodine in desiccated thyroid. The reason for this condition appears to be due to the addition of bromine to a warm alkaline solution with the probable formation of oxidized bromine compounds which in turn liberate iodine in the blank. By modifying the method so that the filtrate is acidified before adding the bromine, neither "slipping" end points nor reagent blank titrations were encountered, and recoveries of iodine in close agreement with theoretical values were obtained. Titrations were conducted at various *p*H values ranging from 1.2 to 3.2.

SEVERAL investigators have noted the returning or "slipping" end point in the various alkaline fusion methods for the determination of iodine in desiccated thyroid. Johnson and Nelson (1) recommend that the final titration be conducted between *p*H limits of 2.2 and 2.5. They state that at *p*H values below 2.0, the rate of return of color increases. Waters and Beal (2) conclude that the return of color results from the use of an excess of bromine, with the possible formation of an oxidized bromine compound which does not dissociate at the *p*H of the titration. These authors also note an increased intensity of color in the reagent blank. They found that if the bromine was added at *p*H values below 4.5, the end point did not fade, but that when the bromine was added to solutions at this *p*H, low results were obtained. They state this condition was caused either by incomplete oxidation or by loss of iodine. Block and Waters (3) report that, regardless of the normality of the thiosulfate solution used, the bromine should be purified before further use if a reagent blank greater than 0.2 cc. is obtained. Karns and Donaldson (4) describe a method for purifying the bromine used in the determination of iodine.

The Methods of Analysis of the Association of Official Agricultural Chemists include the Elmslie-Caldwell method for the determination of iodine (5). This method is, like the U. S. P. XIII method, an alkaline fusion method but differs from the U. S. P. method in that the bromine is added after the filtrate is acidified. With this method slipping end points and reagent blanks are rarely, if ever, encountered. Johnson and Frederick (6) studied the Elmslie-Caldwell method and reported low results when the method was applied to mineral mixtures containing little or no organic matter. When organic matter was added, however, these authors obtained better recoveries of the added iodine.

The present study was undertaken in order to compare the accuracy of the U. S. P. XIII and Elmslie-Caldwell methods and to determine the effect of modifying the U. S. P. XIII method by adding the bromine after the filtrate had been acidified.

EXPERIMENTAL

A mixture composed of casein, starch, and recrystallized *o*-iodobenzoic acid (m. p. 162°) was assayed by the U. S. P. XIII method, by the Elmslie-Caldwell method, and by the U. S. P. XIII method modified by acidifying the filtrate before adding the bromine. Another series of assays, using the same methods, was run on desiccated thryoid powder and on aliquots of a solution composed of reagent grade potassium iodide in distilled water. The *p*H was determined with a glass electrode apparatus prior to the addition of the potassium iodide and starch indicator. All titrations were carried out at 25° ± 1° with *N*/200 thiosulfate. The ignitions were conducted by heating for thirty minutes at 600°, and reagent grade chemicals were used throughout. The bromine was labeled to contain not more than 0.05% iodine and was purified before use according to the method of Karns and Donaldson (4). The results of these experiments are set forth in Tables I, II, and III.

In order to determine the effect of *p*H on the reagent blank, a series of blanks was run at *p*H values ranging from 1.2 to 3.2. The quantities of reagents used were those directed by the U. S. P. XIII with the exception that the phosphoric acid was added until the desired *p*H was obtained. One-half of the blanks were run as directed by the U. S. P. XIII method and in the other half of the blanks, the acid was added before the addition of the bromine. These results are given in Table IV.

DISCUSSION

The results shown in Tables I and II indicate recoveries of iodine in close agreement with the theoretical values. Low results were not obtained when the filtrates were acidified before adding the bromine, although Waters and Beal (2) have reported results as much as 25% low when they carried out the oxidation in this manner. Furthermore, in none of the assays here reported was a reagent blank obtained when the bromine was

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added to the acidified filtrate even though titrations were carried out at *p*H values ranging from 1.2 to 3.2. With the U. S. P. XIII method, however, slowly developing color in the reagent blanks was noted in every instance, and "slipping" end points were frequently encountered. Table III indicates that comparable results are obtained by all three methods in the assay of desiccated thyroid and also shows the slow development of color in the reagent blank in the U. S. P. XIII method.

TABLE I.—RECOVERY OF IODINE FROM POTASSIUM IODIDE SOLUTION

Method	Gm. I Found	Gm. I Added	*p*H	Cc. Blank	Remarks
U. S. P. XIII	0.00186	0.00186	2.2	0.1	Color developed slowly in blank. End point returned slowly
	0.00244	0.00248	2.2	0.2	
	0.00310	0.00296	2.3	0.1	
Modified U. S. P. XIII	0.00217	0.00217	2.2	0.0	No color in blanks after 10 minutes' standing. End point did not return
	0.00244	0.00248	2.3	0.0	
	0.00309	0.00310	2.4	0.0	
Elmslie-Caldwell	0.00245	0.00249	2.8	0.0	No color in blanks after 10 minutes' standing. End point did not return
	0.00305	0.00310	2.7	0.0	
	0.00279	0.00280	2.7	0.0	

TABLE II.—RECOVERY OF IODINE FROM MIXTURE OF *o*-IODOBENZOIC ACID, STARCH AND CASEIN

	Iodine Content 0.0348%			
Method	I Found, %	*p*H	Cc. Blank	Remarks
U. S. P. XIII	0.0338	2.3	0.015 (*p*H 2.2)	Color developed slowly in blank. End point returned slowly
	0.0341	2.2		
	0.0340	2.2		
Modified U. S. P. XIII	0.0341	2.3	0.0 (*p*H 2.2)	No color in blank after 10 minutes' standing. End point did not return
	0.0339	2.3		
	0.0347	2.2		
Elmslie-Caldwell	0.0348	1.6	0.0 (*p*H 2.6)	No color in blank after 10 minutes' standing. End point did not return
	0.0342	3.1		
	0.0338	2.9		

TABLE III.—ASSAY OF DESICCATED THYROID POWDER

Method	I Found, %	*p*H	Cc. Blank	Remarks
U. S. P. XIII	0.224	2.1	0.1 (*p*H 2.2)	Color developed slowly in blank. End point returned slowly
	0.224	2.1		
	0.220	2.2		
	0.225	2.3		
Modified U. S. P. XIII	0.223	2.1	0.0 (*p*H 2.8)	No color in blank after 10 minutes' standing. End point did not return
	0.224	2.2		
	0.220	1.4		
	0.226	3.0		
Elmslie-Caldwell	0.225	2.7	0.0 (*p*H 2.8)	No color in blank after 10 minutes' standing. End point did not return
	0.224	2.6		
	0.222	2.8		
	0.229	2.6		

TABLE IV.—EFFECT OF *p*H ON REAGENT BLANKS

Method	*p*H	Cc. $N/200$ $Na_2S_2O_3$	Remarks
U. S. P. XIII	1.2	0.1	Color developed within one minute of the time the potassium iodide and starch were added
	1.5	0.1	
	1.8	0.2	
	2.0	0.1	
	2.5	0.2	
	3.0	0.1	
	3.2	0.2	
Modified U. S. P. XIII	1.2	0.0	No color developed in this series of blanks even after 10 minutes' standing
	1.5	0.0	
	1.8	0.0	
	2.0	0.0	
	2.5	0.0	
	3.0	0.0	
	3.2	0.0	

In view of the fact that bromine, when added to warm, alkaline solutions, forms hypobromites, bromates, etc., it would appear possible that residual traces of such oxidized bromine compounds could cause the liberation of iodine in the reagent blank. If, however, bromine is added to an acid solution, these oxidized compounds of bromine are not formed.

Baumann and Metzger (7) report that alkali iodides are quite volatile, and that on heating iodides with alkali carbonates at temperatures as low as 85°, serious losses occur. The results shown in Table I do not corroborate their findings.

CONCLUSIONS

1. With the U. S. P. XIII method for the determination of iodine in desiccated thyroid, results very close to the theoretical values are obtained when the alkaline filtrate is made acid before adding the bromine.

2. The proposed modification of the U. S. I XIII method eliminates the blank titration an the "slipping" end point, provided reagent grad chemicals are used throughout the determin tion.

3. "Slipping" end points and development c color in the reagent blanks were not observe with the modified method between *p*H limits c 1.2 and 3.2.

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Electrodialysis in the Field of Antibiotics*

By HENRY FISCHBACH†

THE INTENT of this report is to acquaint other investigators in the field with the value of a well-known technique, electrodialysis, hitherto not used in the study of Streptomycin or Bacitracin. It is known that both Streptomycin salt (1) and Bacitracin (2) will dialyze through a semipermeable membrane. In this laboratory it has been found that both of these antibiotics will migrate toward the cathode when the respective solutions are subjected to a direct current. Electrodialysis incorporates both of the above properties.

During the current investigations there has been a need for concentrating "pooled" mixtures of Streptomycin hydrochlorides and sulfates in the laboratory. It has been found that concentration by electrodialysis results in greater recoveries and requires less time of the investigator than the conventional absorption on Norite A or Alumina with its subsequent filtrations and elutions. Both procedures effect a certain degree of purification dependent on the type of impurities. Similar results are also achieved with Bacitracin. Only one type of electrodialysis used by the author will be dis cussed in this article.

EXPERIMENTAL

Equipment.—The requisite equipment may be o the simplest variety; two cylindrical platinun screen electrodes (50 mm. length x 12 mm. diameter) a glass stirrer, a source of direct current capable o delivering 300 milliamperes at 450 volts, suitabl dialyzing tubing, and a means of reading milli amperes and volts.

Method.—The aqueous sample is placed in a con venient glass container; the two electrodes, 60 to 7 mm. apart, housed in cellophane bags are lowere into the solution along with a stirrer, and the curren adjusted to the desired level. The cellophane bag were made from tubing that was 8.4 cm. in circum ference. Each bag was tied with string to the uppe shaft of the electrode and was punctured at a poin well above the level of the liquid to permit the escap of evolved gases. The cell contents were main tained at 15 to 20° by means of an outer bath. B utilizing a hypodermic syringe and needle, sample may be removed from either the anolyte or catholyt without interrupting the current.

Streptomycin.—Mixtures of Streptomycin sulfat and chloride solutions containing approximatel 1,000,000 units in 200 ml. have been electrodialyze and a recovery of 85–90% achieved in three hour using a current of 240 milliamperes at 430 volts The free base collected in the catholyte was neutra lized with the desired mineral acid. The size of th cellophane bags was such that they convenientl

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† The author wishes to acknowledge the cooperation of the Division of Penicillin Control and Immunology of the Food and Drug Administration for the bioassays.

held 20 to 30 ml. thus affording a seven- to tenfold concentration. When Streptomycin calcium chloride complex was so treated, it was noted that the catholyte took on an amber color during the electrodialysis and the resultant recovery was low (60 to 70%). Apparently the accumulation of calcium hydroxide at the cathode is sufficient to cause some alkaline degradation as evidenced by the appearance of 5% (based on original amount of Streptomycin) free maltol in the catholyte after the electrodialysis of the complex. Preliminary experimentation indicates that the accumulation of alkali and alkaline earth ions in the catholyte may be circumvented by means of a circulating mercury cathode or a cell with several compartments separated by membranes of different porosities. Such devices would be particularly valuable for the direct removal of Streptomycin or Bacitracin from broths. These techniques will be discussed in a later publication.

Bacitracin.—When the Bacitracin is subjected to electrodialysis using a current of 100 milliamperes at 400 volts it appears to migrate to the cathode at least as readily as Streptomycin. This ease of dialysis is not indicative of a high molecular weight polypeptide. By this technique it has been possible to effect a marked increase in the purity of Bacitracin that was available to this laboratory. A sample assaying 37.5 units/mg. was electrodialyzed for four hours during which time the catholyte was syphoned off every hour and replaced with distilled water. The fractions were neutralized with dilute hydrochloric acid and dried from the frozen state. Of the four fractions, the third exhibited the highest potency, 58.6 units/mg., an increase of 55% over the original. Repeating the above fractional electrodialysis with the third fraction as starting material over a three-hour period, furnished a fraction assaying 62.2 units/mg. It is apparent that a cell with several compartments with membranes of different dialysis coefficients, the least permeable surrounding the cathode, would be advantageous. Further, it should be mentioned that the recovery data are significant. When 500 mg. of Bacitracin (37.5 units/mg.) in 200 ml. distilled water are electrodialyzed under the above conditions, 90% of the Bacitracin is recovered in one hour and 95% in two hours.

A unique application of this technique was found in the purification of a sample of Streptomycin which was highly contaminated with pyrogens. Repeated filtrations through Seitz filters failed to remove the pyrogens. Finally, the sample was electrodialyzed. The cellophane bag used for collecting the catholyte was heated overnight at 102°; aseptic pyrogen-free water was used to fill the catholyte bag, and the syringe and glass container for storing the electrodialyzed Streptomycin were pyrogen-free. The resulting assays indicated that the pyrogenic content had been reduced to a negligible level.

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1949 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1949 Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to the Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, Dr. Robert P. Fischelis, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1949 Award, nominations must be received before January 1.

Establishment of the iodine award was first announced at the Milwaukee convention of the AMERICAN PHARMACEUTICAL ASSOCIATION which administers the competition.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During a period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council and which functions under prescribed rules.

Should the award committee fail to find a suitable recipient in any year, two awards may be made during the next year if suitable recipients are selected.

A New Absorption Delaying Vehicle for Penicillin

By F. H. BUCKWALTER and H. L. DICKISON

THE NEED for a repository form of penicillin which could be injected less frequently than aqueous penicillin solutions and still maintain therapeutic blood levels was recognized early.

Although several methods have been proposed (1–10), the most successful, until recently, has been the familiar peanut oil–beeswax formulation introduced by Romansky and Rittman (1). This product utilizes the principle of a delaying vehicle with a soluble penicillin salt.

In our search for a satisfactory repository vehicle, we set up the following characteristics which this vehicle should possess:

1. It must be nontoxic, nonirritating, and nonallergenic.

2. It must not affect adversely the stability of penicillin.

3. It must possess a viscosity to permit withdrawal into a syringe and administration at room temperature.

4. It should retard the rate of release of penicillin from the site of injection.

5. It should be of such consistency as to prevent settling of suspended penicillin particles.

It was found that gels of aluminum stearates and vegetable oils would meet most of the above requirements. The viscosity of those gels will depend on which aluminum stearate (mono, di-, or tri-) is used, the amount of the aluminum stearate, the type of oil, and the conditions under

TABLE I.—BLOOD LEVEL STUDIES IN RABBITS USING SODIUM PENICILLIN G AND PROCAINE PENICILLIN G

Product	Rabbit	Blood Levels, Hr. 1	4	24	28	48	77	96	100	150	174	198	222	242
A	106	..	4.8	0 15	0.04	..	..	..	..	..	....	....	....	..
	107	..	4.4	0.30	0.07	..	..	..	..	..	..	....	....	...
	108	..	4.6	0.18	0 04	..	..	..	..	..	..	....	....	...
	110	..	3 4	0.11	NR	..	..	..	..	..	..	....	....	...
	130	..	5.0	0.10	NR	..	..	..	..	..	..	....	....	...
	131	..	0.8	0.55	0.14	..	..	..	..	..	..	....	....	...
	132	..	2.2	0.21	0.05	..	..	..	..	..	..	....	....	..
	133	..	3.3	0.08	NR	..	..	..	..	..	..	....	....	...
	134	..	2.4	0.04	NR	..	..	..	..	..	..	....	....	...
	135	..	3.8	0.59	0.04	..	..	..	..	..	..	....	....	...
B	166	..	..	0.08	0.04	NR	..	..	..	..	..	....	....	...
	168	..	..	0.56	0.08	NR	..	..	..	..	..	....	....	...
	51	..	4.4	0.15	0.10	NR	..	..	..	..	..	....	....	...
	172	..	2.45	0.22	0.12	0.05	..	..	..	..	..	....	....	...
	173	..	1.55	0.09	0.03	NR	..	..	..	..	..	....	....	...
	174	..	2.45	0.58	0.28	0.13	..	..	..	..	..	....	....	..
	175	..	1.85	0.43	0.06	NR	..	..	..	..	..	....	....	...
	176	..	3.05	0.67	0.07	0.07	..	..	..	..	..	....	....	..
C	372	7.30	2.75	0.03	NR	NR	..	..	..	..	..	....	....	...
	373	4.85	3 55	0.09	0.04	NR	..	..	..	..	..	....	....	...
	374	3.50	2.45	0.24	0.14	NR	..	..	..	..	..	....	....	...
	375	6 50	2.98	<0.01	0.02	NR	..	..	..	..	..	....	....	...
	376	9.85	4.95	NR	NR	0 02	..	..	..	..	..	....	....	...
	377	6.75	3.83	NR	NR	NR	..	..	..	..	..	....	....	...
	378	7.10	3.05	0.40	0.21	NR	..	..	..	..	..	....	....	...
	379	5.40	1.53	0.8	0.11	NR	..	..	..	..	..	....	....	...
	380	7.00	3.10	0 05	<0.01	NR	..	..	..	..	..	....	....	...
	381	6.85	2.65	NR	NR	NR	..	..	..	..	..	....	....	...
D	365	NR	0.85	0.43	0.17	0.15	0.09	..	0.06	..	..	....	....	...
	366	NR	1.05	0.25	0.20	0.18	0.08	..	0.07	..	..	....	....	...
	368	NR	1.1	0.39	0.18	0.17	0.07	..	0.05	..	..	....	....	...
	370	NR	0.78	0.54	0.22	0.09	0.03	..	NR	..	..	....	....	...
	371	NR	3.05	0.45	0.21	0.11	0.05	..	..	..	..	....	....	...
E	382	0.30	0.17	0.18	0.2	0.27	0.20	0.23	..	0.04	0.04	NR	NR	...
	383	0.60	0.22	0.31	0.27	0.42	0.16	0.24	..	0.10	0.09	<0.03	0.03	NR
	384	0.35	0.14	0.19	0.14	0.20	0.06	0.12	..	0.06	0.05	<0.03	<0.03	NR
	385	0.63	0.28	0.25	0.27	0.26	0.24	0.37	..	0.09	Died	....	....	...
	386	0.66	0.25	0.35	0.34	0.32	0.17	0.15	..	0.07	0.06	NR	NR	...
	387	0.27	0.09	0.19	0.18	0.17	0.20	0.20	..	0.11	0.06	0.05	NR	...
	388	0.74	0.18	0.29	0.21	0.23	0.13	0.24	..	0.06	NR	NR	NR	...
	389	0.41	0.15	0.19	0.25	0.20	0.23	0.29	..	0.10	0.06	NR	NR	...
	390	0.86	0.28	0.23	0.3	0.26	0.20	0.25	..	0.06	0.05	NR	NR	...
	391	0.49	0.26	0.3	0.21	0.33	0.25	0.19	..	NR	NR	NR	NR	...

NR—Indicates no zones of inhibition on plates.

	Particle Size
[illegible] penicillin G in peanut oil containing beeswax (Romansky type)	67% over 50 microns
[illegible] penicillin G in peanut oil gelled with 2% aluminum monostearate	70% over 50 microns
peanut oil	93% over 50 microns
peanut oil gelled with 2% aluminum monostearate	79% over 50 microns
peanut oil gelled with 2% aluminum monostearate	98% less than 5 microns

which the gel is made. Since these gels possess a high degree of water repellency, it was reasoned they should be of particular value for parenteral use in delaying the release of suspended penicillin and hence prolonging blood levels. These gels possess thixotropic properties which effectively prevent settling of suspended penicillin particles. No discernible settling is evident after centrifuging five minutes at 1000 r. p. m. Extensive work with these gels indicated they had no detectable effect on the stability of the suspended penicillin salts. Their viscosities were such as to enable easy withdrawal into a syringe and administration at room temperature.

Peanut oil gels containing 2 per cent aluminum monostearate and various penicillin salts were tested in rabbits for blood levels. In each case, the products contained 300,000 units of the penicillin salt per cc. of gel. We have studied in these gels crystalline sodium penicillin G, crystalline potassium penicillin G, calcium penicillin, aluminum penicillin, zirconium penicillin, procaine penicillin G, and various amine penicillins. We have studied the effect of particle size on duration of blood level. The results with large and small procaine penicillin crystals are presented in Table I and Fig. 1. The complete particle size studies with various penicillin salts will be presented in a later communication.

Table I gives the results of blood level studies in rabbits using the sodium penicillin G and procaine penicillin G formulations mentioned above. For comparison, results with sodium penicillin G in peanut oil and beeswax (Romansky type formula), and procaine penicillin G in oil are included. Complete results with all the formulations mentioned above will be presented elsewhere. For these studies, the rabbits were given intramuscular injections of 50,000 units/Kg. into the thigh muscles. We have found that this dosage of 50,000 units per kilogram correlates rather closely with the results obtained by daily intramuscular injections in man of 300,000 units.

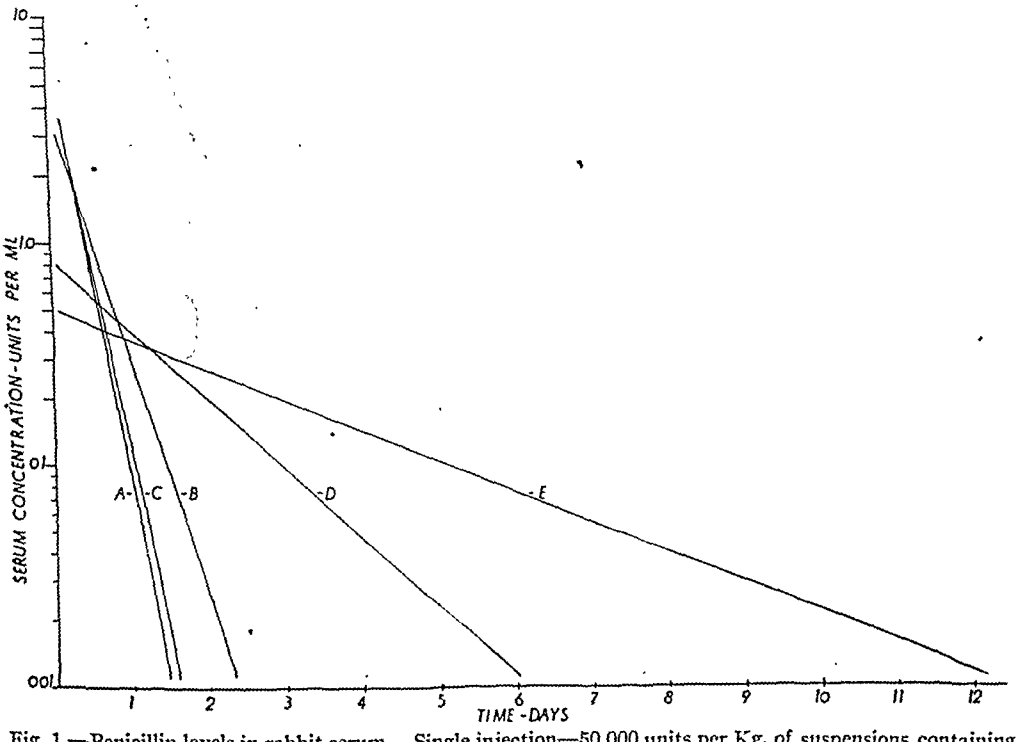

Fig. 1.—Penicillin levels in rabbit serum. Single injection—50,000 units per Kg. of suspensions containing 300,000 units per ml.

A, Sodium penicillin G in peanut oil containing beeswax.
B, Sodium penicillin G in aluminum stearate gel.
C, Procaine penicillin G in peanut oil.
D, Procaine penicillin G in aluminum stearate gel, large particles.
E, Procaine penicillin G in aluminum stearate gel, small particles.

The blood samples were assayed by a cup-plate method employing *Sarcina lutea* as the test organism. Pretreatment blood samples in all instances were negative. The effect of the vehicle is shown graphically in Fig. 1 where average blood levels are plotted against time in days. The submitted data are intended to show the super-

iority of peanut oil–aluminum stearate gels as repository forms for penicillin over peanut oil alone, or peanut oil–beeswax combinations.

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Sunscreen Compounds*

By W. D. KUMLER and T. C. DANIELS

The region in which a compound must absorb radiation to be an effective sunscreen is dependent on the solar radiation curve and the erythema curve. The ordinates of these curves were multiplied in the region where they overlap to obtain what we have chosen to call the "Sunburn Curve." This ranges from 2900 to 3260 Angstroms and has a maximum at 3080 Angstroms. Compounds with extinction coefficients two to eight times higher in this region than those of compounds commonly used as sunscreens are described.

AGENTS employed for protection against solar radiation have been divided into two general types, the physical and the chemical. The physical type contains such agents as zinc oxide or titanium oxide which are spread on the skin in a fine state of subdivision and by their opacity and high reflecting power prevent most of the light from reaching the skin. These compounds give the skin an artificial appearance and because of this are not considered desirable. We are concerned here only with the chemical sunscreen type which may be colorless and transparent to visible rays of sunlight but is opaque to the rays causing sunburn. These compounds may be applied as solutions, as lotions, or as ointments to leave a film that is not noticeable but which is effective in preventing sunburn.

The ideal sunscreen compound has to meet a wide variety of specifications

1. It must absorb or filter out the rays causing sunburn which are those in the region from 2900 to 3300 Angstroms.

2. It should be stable in the presence of light, air, and moisture, or if it is decomposed under these conditions, the decomposition products should have comparable absorption to the ori inal compound in the 2900- to 3300-Angstro region.

3. It should have very slight or no absorptic for the long ultraviolet rays beyond 3400 An stroms which are thought to produce tannir without appreciable erythema.

4. The compound and decomposition proc ucts which may be produced under conditior of use should be nontoxic and nonirritating.

5. It should be nearly neutral so untowar effects are not produced by the presence of aci or base on the skin.

6. It should have good solubility in the oint ment base or vehicle in which it is to be formu lated and should have low water solubility t prevent rapid removal by perspiration.

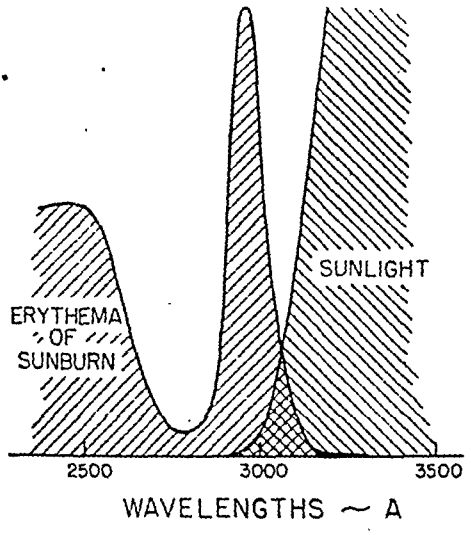
7. It should be relatively nonvolatile so it wi not evaporate under conditions of use.

8. It should not be rapidly absorbed throug the skin.

Two factors determine where a compoun should absorb light to be an effective sunscreer The first is the relative amount of erythema prc duced by various wave lengths of light, and th second is the energy distribution in the sunligh reaching the earth. Figure 1 shows the erythema wave length curve (1) and the energy distributio of sunlight–wave length curve (2). It is apparen that man needs little or no protection below 290 Angstroms. Although wave lengths around 250 Angstroms are effective in producing erythem there are no rays shorter than 2900 Angstrom reaching the earth. The shorter wave length are screened out by the atmosphere. On th other hand, although the energy reaching th earth at wave lengths longer than 3300 is ver high, one need not be protected from such ray

* Received August 11, 1948, from the College of Pharmacy, University of California, San Francisco, Calif.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.

because they do not produce erythema in the normal person. In cases of people suffering from *Xeroderma pigmentosum*, *Hematoporphyria*, or *Hydroa aestivale* it is desirable to prepare special screens that absorb light of longer wave lengths as well as those in the erythema range. Normally such preparations will contain more than one absorbing agent. On the other hand, a screen to protect from the rays of a mercury vapor sterilization lamp, which yields over 80 per cent of its energy at 2537 Angstroms, requires a compound with high absorption at these short wave lengths. At present we will not consider these special cases but confine the discussion to sunscreens for the normal person.



From Coblentz and Stair, J. Research National Bureau of Standards

Fig. 1.—Erythema-wave length and sunlight energy-wave length curves.

Since the amount of sunburn produced by a given wave length of light is as a first approximation the product of the amount of light of that wave length reaching the skin times the relative effectiveness of that wave length in producing erythema, we have multiplied the ordinates of the erythema curve by those of the sunlight energy distribution curve and thus obtained the "Sunburn Curve" which is represented by the dotted line in Fig. 2. This curve shows graphically the wave lengths that should be screened out to prevent sunburn, and the height of the curve at any point gives an approximate indication of the relative importance of the various wave lengths in producing erythema.

The peak of this curve is at 3080 Angstroms. The higher the extinction coefficient of a compound at this wave length the greater will be the effectiveness of the compound as a sunburn preventive. In this respect the mono- and di-alkyl-para-aminobenzoate esters have very favorable extinction coefficients compared with compounds commonly used as sunscreens. Figure 2 shows the propitious relation of the extinction coefficient-wave length curves of these compounds to the "Sunburn Curve." These compounds have five to eight times the screening power of the salicylates and anthranilates, three times that of the umbelliferones and about twice that of the unsubstituted para-aminobenzoates. Ethyl-*p*-ethylaminobenzoate, which is an example of a mono-substituted compound, has an extinction

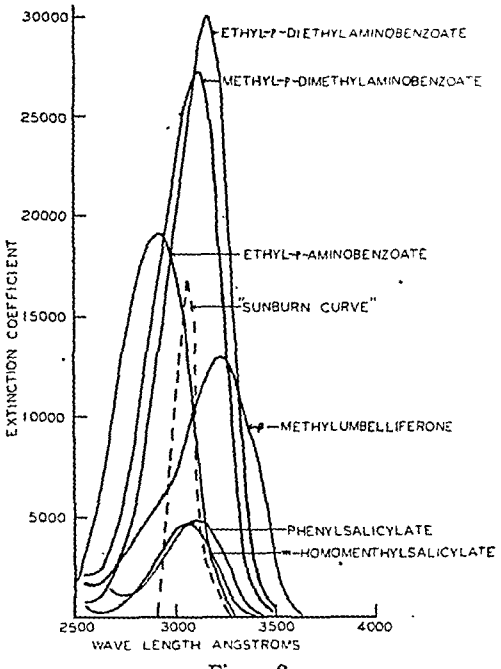


Figure 2.

coefficient of 23,200 at 3050 Angstroms which is very close to the maximum of the "Sunburn Curve." This compound does not have quite as high an extinction in this region, however, as some of the dialkylaminobenzoates. The latter compounds meet favorably the other specifications for an ideal sunscreen compound

The dialkylaminobenzoate esters have good stability in the presence of light and air. This property is not shown by the salicylates or umbelliferones which have free phenolic hydroxy-groups, or by the anthranilates and *p*-aminobenzoates which have free aromatic amino groups. It is reported in the literature (3) that both *p*-aminobenzoates and anthranilates discolor in sunlight and that a solution of menthyl salicylate retains only 50 per cent of its screening ability

after an exposure of four hours to the sun's rays. A solution of methyl-para-dimethylaminobenzoate contained in a Vycor tube remains colorless after being exposed four and a half hours either to sunlight or to the light of a mercury vapor lamp. The original optical density of the solution was 1.70 and after an exposure of four and a half hours one-half inch from a 100-watt mercury vapor lamp the density was unchanged. After exposure to direct sunlight from 11:40 A.M. to 4:20 P.M. the density was 1.65, only three per cent less than the original density, and this difference is within the experimental error of the measurements.

The para-dialkylaminobenzoate esters meet the other requirements for good sunscreen compounds. They are nearly neutral, are nonvolatile, have good solubility in the vehicle or ointment base ingredients, have low water solubility, have no absorption beyond 3500 Angstroms and very little beyond 3400 Angstroms, and are relatively nontoxic and nonirritating.

CONCLUSIONS

A "Sunburn Curve" has been calculated by multiplying the ordinates of the erythema curve by the ordinates of the sunlight energy distribution curve. The height of this curve gives an indication of the relative importance of various wave lengths in causing sunburn. The properties of an ideal sunscreen compound are described. The ultraviolet absorption spectrum of some mono- and dialkyl-para-aminobenzoic acid esters has been measured. These compounds are more effective sunscreens than the compounds commonly employed for this purpose. They are also quite stable in the presence of light and otherwise closely approach the requirements for an ideal sunscreen.

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United States Pharmacopœial Convention

Eighth Annual Financial Statement, Covering the Period from May 1, 1947 to April 30, 1948, and Based Upon the Report of the Auditor

STATEMENT OF INCOME AND EXPENSE

Income			
Sale of Pharmacopœias:			
Collections		$336,724.71	
Add: Increase in Accounts Receivable			
April 30, 1948	$20,609.74		
April 30, 1947	890.75	19,718.99	$356,443.70
Sale of Reference Standards, A. M. A. Articles, etc.:			
Collections		$ 13,711.45	
Add: Increase in Accounts Receivable			
April 30, 1948	$ 1,787.20		
April 30, 1947	803.00	984.20	
		$ 14,695.65	
Add: Increase in Collections Pending Remittance to the Treasurer			
April 30, 1948	$ 696.75		
April 30, 1947	487.75	209.00	14,904.65
Interest on Investments:			
Collections	$ 2,600.00		
Accrued Interest	1,242.31	$ 3,842.31	
Less: Amortization of Bond Premiums		88.21	3,754.10

Miscellaneous Income:				
Collections				2,473.80
Total Income				$377,576.25
Less—Expense				
Publication and Sales Expense		$90,044.64		
Less: Increase in Inventory				
April 30, 1948	$14,155.07			
April 30, 1947	6,475.99	7,679.08	$ 82,365.56	
Administration			5,029.28	
Revision		$68,119.21		
Less: Increase in Reference Standard Inventory				
April 30, 1948	$10,139.13			
April 30, 1947	6,033.75	4,105.38	64,013.83	
Research			11,358.62	
Convention			31.49	
Stationery and Supplies—Inventory Adjustment (Minus)			252.46	
Provision for Depreciation:				
Building		$ 346.71		
Furnishings and Equipment		359.45	706.16	
Loss on Disposal of Furnishings and Equipment			44.38	163,296.86
Excess of Income Over Expense For the year ended April 30, 1948				$214,279.39

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS

Cash on Deposit, April 30, 1947		$ 47,646.56
Add—Receipts		
Sale of Pharmacopœias	$336,724.71	
Sale of Reference Standards, A. M. A. Articles, etc.	13,711.45	
Interest on Investments	2,600.00	
Use of Text by Others	2,140.00	
Miscellaneous (Including Rental of Office Space by Remington's Practice of Pharmacy—$300.00)	342.42	355,518.58
		$403,165.14

Less—Disbursements

	Publications and Sales	Administration	Revision	Research	Convention	Total
Printing and Binding	$90,044.64	.....	.....	.....	.....	$ 90,044.64
Salaries	.....	$1,500.00	$13,500.00	$ 6,908.31	.....	21,908.31
Honoraria	.....	200.00	21,700.00	.....	.....	21,900.00
Clerical	.....	1,248.00	8,518.20	151.65	$ 3.06	9,920.91
Meetings	.....	814.96	9,178.83	3,346.93	.....	13,340.72
Supplies	.....	65.50	1,372 58	886.61	.....	2,324.69
Postage and Telegrams	.....	196.73	1,328.15	32.12	28.43	1,585.43
Headquarters	.....	.....	1,447.18	.....	.....	1,447.18
General	.....	1,004.09	11,074.27	33.00	.....	12,111.36
	$90,044.64	$5,029.28	$68,119 21	$11,358.62	$31.49	$174,583.24

Add:		
Purchase of Furnishings and Equipment	$ 942.20	
Investments	150,652.69	326,178.13
Cash on Deposit, April 30, 1948		$ 76,987.01

A MORE DETAILED AND SUPPLEMENTARY STATEMENT OF THE REVISION AND RESEARCH EXPENSES

REVISION—MEETINGS

Conferences—Including Transportation, Hotel, Meals, etc., for Conferences Called or Made by the Chairman	$ 929.16
Meeting of the Revision Committee at Mount Pocono, the Scope Committee at New York City, and the Executive Committee at Philadelphia	8,249.[illegible]
	$ 9,178

REVISION—SUPPLIES

Stationery	$ 227
Mimeograph	606
Reproduction of Scope Voting Sheets for Bulletins	265
Miscellaneous	274
	$ 1,372

REVISION—HEADQUARTERS

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	$4,450.31	$6,908 31

LIBRA

Note on the Microcrystallographic Properties of Rutin, Quercitrin and Quercetin*

By GEORGE L. KEENAN†, ‡

RUTIN, $C_{27}H_{30}O_{16}.2H_2O$, a glycosidic principle, is found in rue (*Ruta graveolens*,L.) and many other plants, and is of interest in that it is supposed to have an important effect on resolving arteriosclerosis and other functions of assimilation. The lack of microscopic data on the substance initiated the present study for purposes of record, also including a similar investigation in respect to quercitrin and the flavone, quercetin, the latter being one of the products of hydrolyzing both rutin and quercitrin.

EXPERIMENTAL

Rutin is lemon-yellow and the microscopic examination in ordinary light shows that it consists of very minute rods, almost too small for satisfactory inspection. Therefore, it was found more practicable to crystallize the material from alcohol, thus obtaining larger rods. Rutin crystallizes with 1 mol. of solvent of crystallization from alcohol. With crossed nicols, the extinction is parallel and the sign of elongation is negative. The rods were not large enough to reveal interference figures with the conoscopic examination. The significant refractive indices are: $\alpha = 1.508$ (commonly shown lengthwise on rods), $\beta = 1.734$ (commonly shown crosswise and apparently the β-value although an interference figure could not be obtained) $\gamma = >1.734$. All ± 0.002.

Quercitrin, $C_{21}H_{20}O_{11}.2H_2O$ is the glycoside found in the inner bark of the black oak (*Quercus velutina*, Lam.) and other plants. It is orange-yellow and on hydrolyzing, quercetin, in addition to rhamnose, is formed. In ordinary light, the microscopic examination shows that the material is characterized by thin plates, many of which are elongated. These thin plates give a glistening effect to the powder. With crossed nicols, the extinction is parallel, the plates invariably extinguishing sharply, therefore precluding any interference figures with the conoscopic examination. The significant refractive indices are: $\omega = 1.508$ (frequently shown on the elongated plates when their long axis is parallel to the vibration plane of the lower nicol), $\epsilon = >1.734$. Both ± 0.002.

Quercetin, $C_{15}H_{10}O_7.2H_2O$, the aglucone of rutin, is yellow and consists of clusters of spindle-shaped crystals. These clusters are extremely small and best observed in ordinary light at high magnification (4 mm. objective and 10× ocular found satisfactory). Broken fragments of the spindle shaped crystals are frequently observed in the preparation. With crossed nicols the spindle-shaped crystals extinguish sharply when their long axis is parallel to the plane of vibration of the lower nicol (lengthwise). The sign of elongation is negative. The crystals are too small to show interference figures with the conoscopic examination. The significant refractive indices are: $\alpha = 1.555$ (frequently shown when the spindle-shaped crystal or fragment is oriented with its long axis parallel to the vibration plane of the lower nicol), $\beta = 1.734$ (not at all common and apparently shown when the spindles are tipped on edge), $\gamma = >1.734$. All ± 0.002.

* Received March 22, 1948.

† Present address: 165 Westwood Drive, Strongsville, Ohio.

‡ The write [illegible] operation of Dr. James F. [illegible] Laboratory, U. S. Departr [illegible] Pa., in furnishing the m [illegible] of Baldwin-Wallace College, Berea, Ohio.

Book Reviews

Law of Drugs and Druggists. 3rd ed. By WILLIAM R. ARTHUR. West Publishing Company, St. Paul, Minn., 1947. xxxvii + 702 pp. 15 x 23 cm. Price $5.00.

Books on pharmaceutical law generally fall into two classes: those written by lawyers who have developed a special interest in pharmaceutical law, and those written by pharmacists who have developed an interest in jurisprudence. The former tend to be heavy with legal terminology and lacking in pharmaceutical applications, while the latter, avoiding these two disadvantages, tend to contribute insufficient legal material to qualify as authoritative works on pharmaceutical law.

"Law of Drugs and Druggists," while definitely belonging to the first of these two classes, is the best approach to a pharmaceutical explanation of the laws pertaining to pharmacy which has appeared to date. The legal language is there, but the book includes a good glossary of the legal terms. The large number of cases used to illustrate the legal topics are all pharmaceutically pertinent or are determining cases related to the origin of the rules of law. There still remains some slight lack of pharmaceutical emphasis, however. For example, while the book is intended for "retail, wholesale and manufacturing druggists" as well as students of pharmacy, the treatment of alcohol is restricted to the legal problems arising from its use in filling prescriptions, to the neglect of considerations arising from its use in manufacturing (at the retail, hospital, or industrial level), or of considerations of the Federal alcohol tax and its draw-back provisions. This is perhaps understandable, however, as too great a degree of completeness would make the book unwieldy to use, in view of the large amount of legal material to be covered.

Pharmaceutical law is, to some extent, criminal law. It includes some principles of tort law. Pharmacists are controlled in the practice of their profession by 49 constitutions, a Federal and a state system of laws, as well as a host of additional local regulations. There is also, in most states, the common law, "a system of principles and rules enunciated by the courts in conformity with immemorial custom and usage." There has also developed a large body of administrative law based upon the decisions of quasi-judicial bodies such as the state boards, the Federal Food Drug and Cosmetic Administration, the Federal Trade Commission and others. There are thousands of court decisions affecting the drug business.

"Law of Drugs and Druggists" is perhaps the only book which attempts to deal with the entire subject of pharmaceutical law, and it approaches the task in a very effective manner. The various legal topics, as they are taken up, are illustrated by digests of actual cases, rather than by discussion. This device has the disadvantage of making the book appear to a great extent to consist of excerpts or quotations from statutes and court decisions. On the other hand, in addition to being concise, it has the great advantage of giving basic material of primary authority—a statement of what the law is rather than what some commentator thinks about it. However, it causes the book to take on some of the characteristics of a reference work. While this is excellent for the pharmacist and teacher, it does increase the difficulty of the student attempting to use the work as a textbook.

The third edition is greatly enlarged and expanded over the previous one. Additional cases have been added and it has been brought up to date on laws governing the vitamins, on the administrative provisions under the Food, Drug, and Cosmetic Act, the Wheeler-Lea Act and others. Over 225 digests of cases and nearly 500 portions of other cases are used to clarify specific points under definite topics. In addition there are over 425 reading references for further material on specific topics. Questions on the cases used are also included for the convenience of teacher and student. Further aids for the student, in addition to the glossary of legal terms, include a chapter on special rules of law as they apply to drug cases, a chapter on special problems of the druggist, an explanation of the different kinds of courts and their jurisdictions, and an explanation of the legal reporter systems and their reports.

A wealth of basic material is presented in this book.—STEPHEN WILSON.

The Chemical Senses. By R. W. MONCRIEFF. John Wiley and Sons, New York, 1946. vii + 424 pp. 14 x 21.5 cm. Price $4.50.

The special senses are physiologically characterized according to their typical responses to mechanical, physical, and chemical stimuli. Many tests are available which describe these senses from the physiological approach. This monograph by Moncrieff is a chemical approach to the senses of smell and taste, which are responsive to external chemical stimuli.

The author introduces the reader to this subject through an introductory chapter on the role of the chemical senses in life in general. This is followed by the anatomic functional physiology of the sense organs proceeding from the general to the specific and including the chemical sensibility of lower animals.

Various schemes for classifying odors are outlined in a short chapter. It is noteworthy that at least one of these schemes is capable of semiquantitative expression.

Two chapters on chemical constitution in its relation to odor and taste will be of special interest to the organic chemist.

Physical properties of odorous material, theories of odor, a chapter on perfumes and essences and one on flavor and food are also covered.

The book is well documented, the references appearing throughout the text rather than segregated. It is well indexed also. While the book often leaves something to be desired in the practical translation [illegible] use, it is a helpful

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Berzelius and Pharmacy*

By GEORGE URDANG†

IT WAS a century ago, on the seventh of August, 1848, that scientists all over the civilized world mourned the death of one of the greatest among the many excellent chemists who, at the end of the eighteenth and during the first half of the nineteenth centuries, systematized and utilized the new views opened by Antoine Laurent Lavoisier (1743–1794). This man was the Swede, Jöns Jacob Berzelius (1779–1848).

This is not the place to list in detail the numerous contributions to chemistry which have earned immortality for the name of Berzelius. It may suffice to state that it was not only investigations based on thoughts of his own, comprehensive and of highest importance as they were, but his evaluation, classification, and supplementation of the chemical findings of his contemporaries which made the work of Berzelius so enormously influential and consequential.

Some Contributions of Berzelius.—On the basis of the suggestions presented by the Frenchmen J. H. Hassenfratz (1755–1827) and P. A. Adet (1763–1832) the great Swede created the chemical symbols which, with some changes, are still in use. He coined the terms allotropy and catalysis, thus harnessing the phenomena concerned into well-defined limits and chemical tools. He investigated the chemical proportions in a way never surpassed

Jöns Jacob Berzelius (1779–1848)

* Presented before the joint session of the A. PH. A. Sec- [illegible] the American Institute of [illegible] Pharmacy.

and proved the validity of the law of proportions not only for inorganic, but also for organic substances. Together with W. Hisinger (1766–1852) he presented the fundamentals of the electrochemical theory more than four years before Humphry Davy (1778–1829) published his epoch-making respective observations.

Pharmaceutical Scientists about 1800.—The chemical period into which Berzelius was born was decidedly influenced by great pharmaceutical scientists. In his own country, Sweden, it had been the apothecary-chemist, Carl Wilhelm Scheele (1742–1786), whose early death had prematurely closed an unusually brilliant chain of discoveries. In France, Louis Nicolas Vauquelin (1763–1829), likewise growing into research from the ranks of pharmacy, was president of the Parisian Academy of Science when Berzelius, in 1819, in a special session of this famous institution presented the French edition of his book on the electrochemical theories and chemical proportions, and Berzelius proudly reports in his autobiographical notes[1] the compliments paid to him by this great apothecary. In Germany, the chemical scene in the late eighteenth and early nineteenth centuries was dominated by the pharmacist, Martin Heinrich Klaproth (1743–1817), whom Berzelius, in his reference to a priority quarrel concerning the discovery of cerium, called "Europe's greatest analytical chemist."[2] There was, furthermore, quite a galaxy of other prominent apothecary-chemists around 1800 everywhere, especially in France and Germany, but also in England and in Russia.

This situation offers an explanation for the fact that, whenever there seemed to be a vague possibility of a practical pharmaceutical activity for the youth Berzelius, it was seized and dwelt upon, and that statements to this effect have been made now and again.

It was in order to contradict the idea of Berzelius having been a pharmacist that Theodor Husemann (1833–1901) in an article written on the occasion of the fiftieth anniversary of the death of the great chemist made the following statements:

"Berzelius, unlike several other famous northern naturalists...was not a pharmacist by profession. He was a physician and, after having completed his studies, he has indeed practiced medicine (as physician at Werner's mineral water establishment at Stockholm) from 1803 to 1806 besides acting as an 'adjunct to the professor of medicine and pharmacy,' a position with an entirely insufficient remuneration."[3]

Pharmaceutical Experience of the Youth Berzelius.—However, although Berzelius never was "a pharmacist by profession," were there not indeed relations of a substantial nature entitling pharmacy to claim some modest part in the development of the great scientist? The fact that the brief notes on Berzelius in J. A. Häfliger's "Biographicon" include the remark "worked in a pharmacy"[4] seems to justify such an assumption. It is furthermore underlined by the following passage in H. G. Söderbaum's book on "Berzelius Development and Growth, 1779–1821":

"Idleness was something which the restless nature of Berzelius could stand the least. He longed for taking up experimental work again and in order to satisfy this longing he decided to look for a position in the pharmacy of some neighboring town. At this time Berzelius was the guest of his aunt Bromander at Bromma near Motala. He remembers a relative and namesake who is an apothecary in Jönköping. He applies for a job, receives, however, a negative reply. He is more successful in Vadstena. There he stays in the pharmacy for the rest of the summer (1799) *and takes part in the entire pharmaceutical work.*[5] This episode, as unessential as it may appear as such, is nevertheless of some interest since it furnishes proof for the fact how well Berzelius already at this early time understood how to take advantage of whatever came into his way and to utilize it in one way or another. The insight into the practical part of the profession of pharmacy which he gained in the plain Vadstena apothecary shop was by no means lost. On the contrary, it offered the first incentive to his publication of thirteen years later entitled 'On the Chemical Preparations in the Pharmacopœias of the Various Countries' which made him *a reformer of Swedish pharmacy.*[6] His later activity in this field has, by the way, by no means been without influence on the organization of the public health system in other countries."[7]

It is in all probability these statements which still in 1948, in the most recent Berzelius biography, written by Wilhelm Prandtl, found their repercussion in a passage telling that during the summer vacation in 1799 the student Berzelius went to the country, "where life was less expensive and occasionally money could be earned by teaching or *as a clerk in a pharmacy.*"[8]

Do the real facts conform with the description of this epsiode in Berzelius' life and the conclusions drawn by the enthusiastic biographer H. G. Söderbaum? The view given by the one who ought to know, Berzelius himself, is quite different, and it is not without an ironical connotation that the same man who wrote the flowery story quoted above was bound to bring his hero's contradictory statements to the knowledge of the world. In 1901, only two years after his first book on Berzelius had appeared, H. G. Söderbaum edited for the Royal Swedish Academy of Science the "Autobiographical Notes" of the great chemist presented by the latter to the Academy. In these "Notes" Berzelius describes his vacation activities of the year 1799 as follows:

"I had an uncle, Daniel Berzelius, who was an apothecary in Jonköping, of whom I asked leave to help in the apothecary shop during the summer. In this way I hoped to acquire distinct pharmaceutical experience, but he refused my request. I then accepted the invitation of my aunt, Mrs. Flora Bromander, to spend the summer with her family at

[1] H. G. Söderbaum, J. J. Berzelius, "Autobiographical Notes," translated into English by Olof Larsell, Baltimore 1934, p. 40; Berzelius quotes Vauquelin as follows: "We consider it as only a proper reparation to say to you that, if your and Mr. Hisinger's work on the chemical effects of the electric pile had been known to us when Davy received the great prize from us, we should have divided it between you and him."

[2] *Ibid.*, p. 51.

[3] *Pharm. Ztg.*, 43, 63(1898).

[4] J. A. Häfliger, Biographicon, in A. Tschirch, "Handbuch der Pharmakognosie," ed. 2, Leipzig, 1931/33, vol. 7, part 2.

[5,6] For the italicized statements Söderbaum refers to M. Pontin, "Biographi," p. 205. Magnus Pontin (1781–1858), *since 1835 physician in ordinary to the King of Sweden,* was a lifelong friend and, for some time, collaborator of Berzelius.

[7] H. G. Söderbaum, Berzelius' "Werden und Wachsen 1779–1821," Leipzig, 1899.

[8] Wilhelm Prandtl, "Humphry Davy—J. J. Berzelius," Stuttgart, 1948, p. 122. Prandtl lists Söderbaum's book on Berzelius among his sources.

Bromma near Motala. Bromander was a very industrious man.... He soon found me much too idle which was not to his taste. He sent me, therefore, after a few weeks to Vadstena where I was to live with his mother, a likable but quite elderly lady. I was also to work with apothecary Wessel who, incorrectly, was considered a skillfull chemist. In his pharmacy there was little to do. But there was an Italian who blew glass and made barometers, a good old man named Josua Vaccano. With him I associated myself and learned to blow glass with a burner, as well as to make barometers and thermometers. With this occupation I busied myself at home between the hours of instruction from him. After a month I returned again to Bromma."[9]

This statement in which Berzelius himself stresses the lack of opportunity to do pharmaceutical work in the Vadstena apothecary shop and mentions the information in the art of glass blowing as the main achievement of this vacational month, deprives this epoch in the life of the great chemist of every pharmaceutical significance.

The difference between the real meaning of the episode in Vadstena as to Berzelius' later pharmaceutical knowledge and interests, however, and the interpretation given to it by his biographer offers an excellent example for one of the most frequent sources of mistaken views in so many biographies: the endeavor to construe some causal connection between every event in the life of the hero with all subsequent ones which seem to develop along the same line. In his later life Berzelius took an active and very beneficial interest in pharmacy. In his younger life he had spent some time in a pharmacy. There had to be a causal nexus between these two facts.

It is interesting to note that another and much more important job, done by Berzelius in a pharmacy only one year after his pharmaceutically rather uneventful stay at Vadstena, has not been given much publicity by those eager to establish an early influence of pharmacy on Berzelius. In the summer of 1800 Berzelius, still only a candidate of medicine, held for some months the position of a physician to the poor at Medevi, a then much frequented Swedish spa. He evaporated the water of the various springs and, leaving the examination of the residues for the time after his return to Upsala, he made "gas analyses on the spot in the apothecary's shop."[10] This proves that the pharmacy at Medevi offered the young chemist the adequate opportunity, apparatus, and reagents for his analysis which, by the way, has to be considered as his first scientific accomplishment and served him as basis for his dissertation. There is, however, no evidence for the assumption that this use of the facilities of an apothecary shop for scientific work had any influence on the attitude of Berzelius toward pharmacy.

Besides, there is no necessity for any attempt at finding some more or less farfetched reason for the activities of Berzelius in the interest of pharmacy. It belonged, after all, to his duties as a professor of chemistry and pharmacy to pay attention to the scientific fundamentals of the latter. Another factor was that, as intimated above, at this time chemistry and pharmacy were still to a very great extent undivided.

[9] H. G. Söderbaum, J. J. Berzelius, "Autobiographical Notes," *loc. cit.*, p. 28.
[10] *Ibid.*, p. 31.

When the young student Berzelius applied for admittance into the laboratory of his chemistry professor at Upsala in order to start practical chemical work, he was given "the two thick volumes" of the renowned "Textbook on the Art of Pharmacy" ("Lehrbuch der Apothekerkunst") by the apothecary and professor of pharmacy at Königsberg in Prussia, Carl Gottfried Hagen. The accompanying advice was: "read them first."[11] The two first preparations to be assigned to the ambitious student were undoubtedly of a pharmaceutico-chemical nature, namely, *Colcothar Vitrioli*, an impure iron oxide obtained by heating iron sulfate, and potassium carbonate obtained by heating cream of tartar.

It is a peculiar accident that it was just the pharmaceutical examination to be passed at that time by the Swedish candidates for the degree of Doctor of Medicine which became a cause of great irritation to Berzelius, not because of any fault or deficiency on his part, but because of personal differences among the respective professors at the University of Upsala.

Berzelius the Pharmacy Teacher.—In 1803, only one year after he had met the requirements for the medical doctorate—the official bestowal of the degree did not take place until midsummer 1804—Berzelius was appointed "Adjunct in Medicine and Pharmacy" at the School of Surgery at Stockholm.[12] In this capacity he gave lectures on chemistry to which "the pupils at the School of Surgery... as also the students of pharmacy were invited to listen...."[13] In 1806 he introduced at the College of Medicine at Stockholm "exercises in pharmaceutical[14] and chemical operations similar to those in Upsala." In 1807 Berzelius was named as professor of medicine and pharmacy at the School of Surgery, his duties comprising the teaching of chemistry, general science (called at that time "natural history"), pharmacy, and medicine. In 1809, finally, the instruction pertaining to the professorship held by Berzelius "was divided among three new professors,"[15] leaving to Berzelius only the two disciplines in which he was interested primarily, chemistry first and then, in its relations to this science, pharmacy. In 1811 Berzelius was given a seat and a voice in the Swedish College of Medicine and it is, for the topic of this paper, of interest that he received this appointment in his capacity as professor of pharmacy.

In 1812, one year after the firm establishment of Berzelius as the academic representative of Swedish pharmacy, he published the aforementioned observations "On the Chemical Preparations in the Pharmacopœias of the Various Countries." The subsequent activities of the great Swede in the interest and for the sake of pharmacy not only in his homeland but in the world at large have been described by Husemann as follows:

"In his capacity as a professor of pharmacy Berzelius has helped the Swedish pharmacists not

[11] *Ibid.*, p. 23.
[12] *Ibid.*, p. 38.
[13] *Ibid.*, p. 44. In referring to these lectures Berzelius confessed the following: "I believed, like all beginners, that completeness and thoroughness were the principal things.... The only one to derive any benefit from this was myself, since, as the number of my auditors diminished daily, I recognized that this manner of presentation was not the correct one." (*Ibid.*, p. 168, note 77.)
[14] Erroneously called "pharmacological" by Larsell.
[15] Söderbaum, J. J. Berzelius, "Autobiographical Notes," *loc. cit.*, pp. 66/67.

only through his lectures. It should not be overlooked that he participated to an extremely great extent in the preparation of the Swedish Pharmacopœias which appeared during the time of his active life. Most biographers have silently passed over the fact that the chemical part of the fifth edition of the Swedish Pharmacopoeia is the work of Berzelius. This edition, planned as early as 1793 but not completed until 1817 because of war and political unrest, offers not only an example as to the selection of drugs admitted, but its brevity and preciseness make it undoubtedly the best of all contemporary pharmacopoeias. In the preparation of the next (sixth) edition of this standard, published in 1845, Berzelius has likewise participated, this time as the chairman of a committee of revision especially appointed for this purpose.

"Berzelius has furthermore given vivid support to the establishment of an association of the Swedish pharmacists intended to promote pharmacy as a science. Having shown his appreciation of the beneficial effect of associations as early as 1807 by becoming one of the founders of the Swedish Society of Physicians, he helped in the founding of a similar pharmaceutical group. When in 1820 the newly formed Swedish Pharmaceutical Society under the chairmanship of Carl Göransson created a library and other useful institutions, Berzelius presented the young group with foreign periodicals and other substantial support besides helping by his advice.

"Naturally, more than through these local activities [in the interest of Swedish pharmacy] Berzelius has promoted pharmacy everywhere through his scientific life work and publications."[16]

It is of interest that the fifth edition of the Swedish Pharmacopoeia, the chemical part of which had been prepared by Berzelius, appeared in the same year in which the aforementioned German apothecary-chemist, Martin Heinrich Klaproth, passed away. It had been Klaproth who, together with the pharmacists Sigismund, Friedrich Hermbstädt (1760–1833) and Valentin Rose, Jr. (1762–1807), had made the new views of Lavoisier and the new chemical nomenclature the basis of the chemical part of the *Pharmacopoea Borussica* published in 1799, thus setting an example for his time which was followed everywhere. It deserves mention at this place that after the death of Klaproth "the chair of that prominent chemist and his position as counsellor in the *Obersanitätskollegium* in Berlin" was offered to Berzelius. He declined "this complimentary offer with thanks" since, as he expressed it, "I was indebted to my own native land for a passable and carefree living, with full opportunity to pursue the science to which I belonged...."[17]

Tributes to Berzelius.—The high esteem, the admiration, and the gratitude felt everywhere for the work and the personality of Berzelius expressed itself on the occasion of his death in numerous and detailed necrologies. But a closer examination shows that those published in English language pharmaceutical journals, for instance in the British *Pharm. J. & Trans.*, 8 148(1848–1849) and in the *Amer. J. Pharm.* 21, 189(1849), were reprints of a necrology published in *Lancet*, while the French *J. Pharm. Chim.* presented its readers with the reprint of a "Biographie Chimique" by P. Louyet. It is understood that these necrologies were of a general nature rather than expressing the particular feelings of the pharmaceutical world toward the great deceased.

It was the German pharmacists who conceived and performed a memorial to Berzelius which has to be regarded as one of the most beautiful tributes of gratitude ever offered to a great benefactor by those who consider themselves his beneficiaries. It is simultaneously proof of the wave of liberalism and universalism, expressing itself in the high emphasis placed on intellectual and humanitarian achievements, which swept over the Germany of 1848.

A "General German Pharmaceutical Congress" ("Deutscher Allgemeiner Apotheker Congress") convened at Leipzig on September 12 and 13, 1848, and its chairman, the pharmacist Dr. L. F. Bley, told in his opening address that "the year 1848 carrying with it the dawn of German liberty has been joyfully welcomed by the pharmacists" who hoped for redemption from "the pressure of tutelage and the domination by bureaucrats."[18]

It was on the second day of this Congress that the pharmacist Dr. E. Geffcken of Lübeck read an "Appeal to the German Pharmacists for the Establishment of a Memorial to Berzelius" from which the following significant part may be quoted:

"Berzelius' professorship of chemistry and pharmacy was not restricted to Sweden where the pharmaceutical profession owes primarily to him the dignified position recognized all over the other parts of Europe; he was, if not directly then indirectly, the teacher of all of us. For where in our fatherland can a pharmacist be found, and be it the most simple practitioner, who does not follow in the one way or another the theoretical or practical rules set by Berzelius. Alas, gentlemen, just here in Leipzig we are at a place from where, multiplied by the printing press, the products of Berzelius' mind have been distributed all over Germany, sometimes even earlier than in Sweden. In this respect it may suffic- to point, as offering adequate evidence, to the five c six editions of his comprehensive textbook of chemis try."[19]

Geffcken suggested as a more fitting memorial t Berzelius, to be presented to the Swedish Academy c Science, a wreath of silver oak foliage with as man individual leaves in solid silver as pharmacists wh had attended the 1848 Pharmaceutical Congress a Leipzig.

On August, 7, 1849, Geffcken reported that hi appeal had been successful and his suggestion real ized, and that "the firm Th. Strube and Son i Leipzig had produced a real piece of art." Havin told that the wreath had, on December 11, 1848 been sent to the Swedish Academy of Science to gether with a poem from the pen of Emanuel Geibel,[20] Geffcken concluded as follows:

"The silver wreath was placed on a silver-trimmed black velvet pillow. In a case attached to it were 1. the beautiful poem by Geibel printed with silver letters on black parchment; 2. the presentation

[16] See footnote 3.
[17] Söderbaum, J. J. Berzelius, "Autobiographical Notes," *loc. cit.*, p. 95.

[18] *Arch. Pharm.*, 106, 83(1848).
[19] *Arch. Pharm.*, 106, 117(1848).
[20] Emanuel Geibel (1815–1884) was one of the best-known representatives of German conventional poetry about the middle of the nineteenth century. He advocated the political unification of Germany under an emperor, not as a republic, hence was called the herald of the emperor ("Der Kaiserherold").

document. A letter expressing the gratitude of the Academy of Science at Stockholm has informed us that the wreath has been deposited beneath the picture of Berzelius in the room reserved for the sessions of the academy."[21]

The first paragraph of the letter of thanks mentioned above, and addressed to the "highly esteemed Pharmaceutical Congress at Leipzig," testifies to the respect in which German cultural and scientific achievements and standards were held at this time by the world at large. It reads as follows:

"The Swedish nation as well as all the other nations pursuing scientific aims are since days far remote indebted to Germany's noble and intelligent people for the unveiling of many a scientific truth and for a variety of useful inventions. Sometimes Sweden has had the satisfaction of repaying, in its limits, this debt. In more recent time it was Berzelius who has furnished his fatherland with such an opportunity. The Academy of which he was the most precious adornment, has received the most beautiful proof of appreciation of this fact from one of Germany's most reputed associations."

Geibel's poem was reprinted in three different modifications, in golden as well as silver letters on blue ground, and in black on white glossy paper. All three modifications, sold for the benefit of old meritorious pharmacy clerks, showed the picture of Berzelius enshrined by the wreath of silver oak leaves and followed by the poem which, in free English translation, appears below.

It is significant that the poem pays homage to Berzelius in the most general terms without mentioning pharmacy at all, except in the dedicatory caption. German pharmacy had, in this case, made itself the speaker for German science on the whole.

There cannot be any doubt. The great Swedish chemist and pharmacy have been connected in many and varied ways. It was not accidental happenings in the youth of Berzelius, some more or less ephemeral early "work in a pharmacy," that served as a kind of vantage point. It was the inescapable constellation of cogent circumstances which forced them together, the great chemist and the time-honored profession in which chemistry as a science has found an early place and continuous cultivation.

Pharmacy as an applied science owes Berzelius so much that it is entitled to claim him as one of its Patron Saints on the basis of one of the most elementary human rights: the right of the grateful to express his gratitude.

[21] *Arch. Pharm.*, 110, 127(1849).

"TO THE MEMORY OF THE MASTER IN SCIENCE

J. J. v. BERZELIUS

THE GERMAN PHARMACISTS, 1848

For the immortal wreath on the slumbering head of Berzelius
Germany joins and presents gratefully leaves of her oaks
For him loved Mother Nature. Behold, her veil, the much folded,
Smilingly threw she aside for him, her favorite son.
Graciously taught she him the secret of natural forces
Which she, ready to bless, calmly had kept in her breast.
Now, however, he solved, an enlightened disciple of Isis,
Thousands of riddles, unlocked numberless wonders to us.
As out of barren rock once Moses elicited water,
He out of metal and stone conjured up sources of life.
What, for the sake of mankind, helpful is joining in matter,
What is adversely opposed, what is harmoniously solved,
To the mortals he showed. Then death reluctantly touched him.
Bringing the favorite son back to the mother again.
Sweden weepingly buries the dead, all Europe deplores him,
But posterity's offerings are admiration and thanks."

LIBRARY

CORRECTION

Add to the article entitled "A New Absorption Delaying Vehicle for Penicillin," by F. H. Buckwalter and H. L. Dickison, THIS JOURNAL, 37, 472 (November, 1948) a footnote reading: "Received June 14, 1948, from the Research Laboratories, Bristol Laboratories Inc., Syracuse 1, New York."

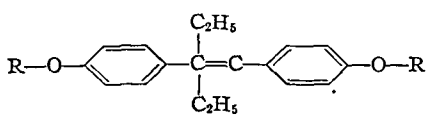
The Antibacterial Action of Basic Ethers of Diethylstilbestrol and 4,4′-Dihydroxydiphenylsulfone*

By D. E. SHAY, G. P. HAGER, L. RICE, and K. DAILEY

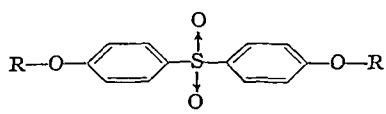
The preparation and the antibacterial activity *in vitro* of five substituted aminoalkyl ethers of diethylstilbestrol on *Staphylococcus aureus* (209), *Eberthella typhosa, Streptococcus pyogenes*, and *Mycobacterium tuberculosis* var. *hominis* and the results of *in vivo* studies of the compounds against *Mycobacterium tuberculosis* using guinea pigs are reported.

Three basic ethers of 4,4′-dihydroxydiphenylsulfone were prepared and tested *in vitro* against *Escherichia coli, Staphylococcus aureus* (209), and *Mycobacterium tuberculosis* var. *hominis* and were found to possess no antibacterial action.

THE diethylaminoethyl ethers of diethylstilbestrol and hexylresorcinol (stilbestryl-bis (2-diethylaminoethyl) ether and hexylresorcinol-bis (2-diethylaminoethyl) ether) have been shown to possess bactericidal action on cultures of *Mycobacterium tuberculosis* var. *hominis* at rather high dilutions (1). Before proceeding to *in vivo* studies using infected animals, it was decided to explore the possibility that the *in vitro* activity might be greater in homologous basic ethers of diethylstilbestrol (I), or in basic ethers of other phenols. It was of particular interest to include in this study basic ethers of 4,4′-dihydroxydiphenylsulfone (II), because of the known antitubercular activity of Promin (sodium 4,4′-diaminodiphenylsulfone-N,N′-didextrose sulfonate)



(I)



(II)

where R = substituted aminoalkyl group.

Attempts to prepare certain of the substituted aminoalkyl ethers by the procedure previously published (1) for preparation of stilbestryl-bis (2-diethylaminoethyl) ether resulted in polymerization of the substituted aminoalkyl chloride. The method was especially unsatisfactory when using 2-(4-morpholino) ethyl chloride which is known to polymerize readily in the methanol used as the solvent (2). It was possible to prepare stilbestryl-bis 2-(4-morpholino) ethyl ether when no polar solvent was used; and much better yields of all the stilbestryl ethers were obtained under these conditions. The disodium salt of diethylstilbestrol was conveniently prepared by reaction of diethylstilbestrol with sodium powdered in xylene. Better results were obtained, however, if the disodium salt was previously prepared by reaction of diethylstilbestrol with a solution of sodium ethoxide in alcohol followed by precipitation with ether and drying. This substance reacted readily with the basic alkyl halides when a mixture of the two in xylene was refluxed for a short time. The same procedure was quite satisfactory for preparation of certain basic ethers of 4,4′-dihydroxydiphenylsulfone.

Reports on the antibacterial properties of diethylstilbestrol and related compounds have appeared from time to time. Russ and Collins used diethylstilbestrol successfully in the treatment of twenty-five cases of prepuberal vulvovaginitis (3). The effectiveness in such infections might be due to the estrogenic activity of the compounds. Faulkner showed that diethylstilbestrol and 4-hydroxy-α,α'-diethylstilbene possess bactericidal activity when tested on *Mycobacterium tuberculosis in vitro* (5).

Substituted aminoalkyl ethers of other phenolic compounds have been tested for antitubercular activity. Some of the diethylaminoalkyl ethers of triiodophenol were found by Saz, Johnston, Burger, and Bernheim to be bactericidal in their action on human and bovine tubercle bacilli *in vitro* (4). It was found that oxygen uptake by the bacilli was decreased, growth *in vitro* inhibited, and the formation of tubercules in the omentum of infected guinea pigs reduced by several of the compounds tested.

Burger, Wilson, Brindley, and Bernheim synthesized several dialkylaminoalkyl ethers of iodinated phenols with variation in the structure of the ether chain (6). The ethers with a 2-methylpiperidinopropyl radical were highly effective on the tubercle bacilli. Activity and toxicity in guinea pigs were usually greater in dialkylaminopropyl than in the corresponding dialkylamino-

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Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.

ethyl ethers. On the other hand, shortening of the ethylene group as in a trihalogenophenoxydiethylaminomethane derivative, caused complete loss of antitubercular action.

Replacement of nuclear iodine by other halogens did not lead to a definite pattern correlating chemical structure to antitubercular activity. Nonhalogenated dialkylaminoalkyl phenyl ethers were found to lack antitubercular properties.

The recent investigations of Chapman, Hager, and Shay (1) revealed the antibacterial properties of stilbestryl-bis (diethylaminoethyl) ether dihydrochloride for *Mycobacterium tuberculosis* var. *hominis*, *Staphylococcus aureus* (209), and *Eberthella typhosa* when tested by a modified Food and Drug Administration procedure (8). The favorable results obtained by these workers was the stimulus for further studies with similar compounds.

The results of the *in vitro* antibacterial tests of five stilbestrol derivatives on *S. aureus* (209), *E. typhosa*, *Strept. pyogenes*, and *M. tuberculosis* var. *hominis* are reported. *In vivo* studies with *M. tuberculosis* var. *hominis* using guinea pigs are also included.

A preliminary investigation of basic ethers of 4,4′-dihydroxydiphenylsulfone was carried out in a manner similar to that used in the study of the stilbestryl ethers.

EXPERIMENTAL

Basic Ethers of Diethylstilbestrol.—A slight modification in the procedure previously reported (1) for the preparation of stilbestryl-bis(2-diethylaminoethyl) ether made possible the production of this compound in greater yields and was applicable to the preparation of other related compounds which could not be prepared satisfactorily by the original procedure.

The disodium salt (67 Gm., 0.25 mol.) of diethylstilbestrol was prepared by dissolving diethylstilbestrol in a solution of sodium (11.5 Gm., 0.5 equivalent) in absolute ethanol (250 ml.). After refluxing for thirty minutes, the disodium salt of diethylstilbestrol was obtained as a grayish white solid by removal of part of the alcohol by distillation under reduced pressure followed by precipitation with ether.

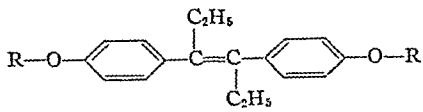
A mixture of the disodium salt of diethylstilbestrol (0.05 mol.), the substituted aminoalkyl chloride (0.2 mol.), and xylene (75 ml.) was refluxed for one to four hours until the suspension of fluffy amorphous solid had changed to a granular, crystalline precipitate of sodium chloride. The reaction mixture was cooled, diluted with an equal volume of ether and extracted with 10% hydrochloric acid. After washing the hydrochloric acid solutions with ether, the free base was precipitated by the addition of 20% sodium hydroxide solution and was then extracted with ether. The ether solution was washed with 20% sodium hydroxide solution to remove any phenolic substances and dried with anhydrous magnesium sulfate. The hydrochloride of the basic ether was prepared from the ether solution in the usual way and purified by repeated recrystallization from mixtures of absolute ethanol and ethyl acetate, ether or acetone.

The compounds prepared by this method are listed in Table I together with pertinent data.

Basic Ethers of 4,4′-Dihydroxydiphenylsulfone.—4,4′-Dihydroxydiphenylsulfone was prepared by treatment of phenol with fuming sulfuric acid (7). The disodium salt was prepared by dissolving 4,4′-dihydroxydiphenylsulfone (25 Gm., 0.1 mol.) in a solution of sodium ethoxide (0.2 mol.) in absolute ethanol (250 ml.). After heating on a steam bath for thirty minutes, treating with charcoal, filtering, and distilling off most of the alcohol under reduced pressure, ether was added and the precipitated white solid was filtered and dried.

A mixture of the disodium salt of 4,4′-dihydroxydiphenylsulfone (0.05 mol.), the substituted aminoalkyl chloride (0.15 mol.), and xylene (75 ml.) was refluxed for two hours. The free basic ethers were recovered from the reaction mixture as directed in the procedure for preparation of the stilbestryl ethers. The hydrochlorides could be prepared only as oils or amorphous solids. The picrates were the only salts that could be obtained in crystalline form, Table II.

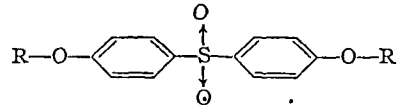
TABLE I.—BASIC ETHERS OF DIETHYLSTILBESTROL



R	M. P. of Dihydrochloride °C.	Yield, %	Formula	Analysis, % Calcd.	Found
2-Diethylaminoethyl-	238–239	57	$C_{30}H_{48}Cl_2N_2O_2$	N, 5.19	5.05
2-Diethylaminopropyl-	(Oil)	..	$C_{44}H_{56}N_8O_{16}$[a]	N, 11.76	11.72
3-Diethylaminopropyl-	215–215.5	55	$C_{32}H_{52}Cl_2N_2O_2$	N, 4.93	4.73
2-(4-Morpholino)ethyl-[b]	255–256	40	$C_{30}H_{44}Cl_2N_2O_4$	N, 4.94	4.61
3-(2-Methylpiperidino) propyl-[c]	232–233.5	29	$C_{36}H_{56}Cl_2N_2O_2$	N, 4.52	4.20

[a] Picrate (m. p. 194–6°).
[b] Morpholine ethanol supplied by Carbide and Carbon Chemicals Corporation.
[c] 3-(2-Methylpiperidino)-1-propanol supplied by Eli Lilly and Company.

TABLE II.—BASIC ETHERS OF 4,4'-DIHYDROXYDIPHENYLSULFONE



R	M. P. of Picrate, °C.	Formula of Picrate	Analysis % Calcd.	Found
2-Diethylaminoethyl-	...	$C_{36}H_{42}N_8O_{18}S$	N, 12.35	12.17
3-Diethylaminopropyl-	165–169	$C_{26}H_{42}Cl_2N_2O_4S$[a]	N, 5.09	4.88
3-(2-Methylpiperidino) propyl-	206–207	$C_{42}H_{50}N_8O_{16}S$	N, 11.35	11.15

[a] Dihydrochloride.

BACTERIOLOGY

Stock solutions (1:100) for performing *in vitro* studies on *S. aureus*, *E. typhosa*, and *Strep. pyogenes* were prepared. From these solutions, dilutions (1:1000; 1:2500; 1:5000; 1:10,000; 1:20,000) of each compound were made. For each compound four sets of five tubes each containing 10 ml. of each of the above dilutions were prepared and sterilized in the autoclave.

For the *in vitro* studies on *M. tuberculosis*, two sets of five dilutions (1:10,000; 1:30,000; 1:50,000; 1:70,000; 1:100,000) of each compound were prepared.

Solutions for study of the *in vivo* antibacterial action of these compounds against *M. tuberculosis* were prepared as follows: 6 ml. of the 1:100 stock solutions of each compound was pipetted into large 250-ml. graduates and normal saline was added to give a total volume of 200 ml. These solutions were then transferred to 250-ml. Erlenmeyer flasks, resulting in a concentration of 150 μg. of each compound per 1 ml. of solution. To prepare a 300-μg. dose per 1 ml., 12 ml. of the 1:100 stock solutions of each compound was pipetted into large 250-ml. graduates and normal saline was added to give a total volume of 200 ml.; these solutions were then transferred to 250-ml. Erlenmeyer flasks, closed with rubber stoppers, and sterilized at fifteen pounds pressure (121°) for fifteen minutes.

The *p*H ranges of the various solutions as determined by means of the Beckman *p*H meter are given in Table III.

The culture medium selected for the tests on *S. aureus* and *E. typhosa* was nutrient agar. This was prepared according to the A. P. H. A. standard method. The medium selected for *Strep. pyogenes* was blood agar prepared in the usual manner. The medium selected for the *in vitro* studies of *M. tuberculosis* var. *hominis* was Petragnani's medium.

The organisms *S. aureus*, *E. typhosa*, and *Strep. pyogenes* were transferred on five successive days preceding the day on which they were to be used in the experiment.

IN VITRO TESTS

The *in vitro* test employed was a modified F. D. A. method of determining phenol coefficients. This modification consisted of a change in the time intervals from one, ten, and fifteen minutes to one, ten, and sixty minutes, respectively. Twenty-four hours before the tests were to be run, a standard 4-mm. loopful of a culture of each organism was transferred into separate sterile tubes containing 10 ml. of nutrient broth and incubated at 37°. After incubation for twenty-four hours bacterial cell counts were made. These results are given in Table III. To one set of dilutions of the compound, 0.5 ml. of the standardized broth culture was added. At intervals of one, ten, and sixty minutes, 1 ml. of each dilution of the compound containing the organism was plated. The plates were incubated at 37° for forty-eight hours. The results of the antibacterial tests are tabulated in Table III. This procedure was followed with each of the five compounds.

The tests on *Strep. pyogenes* were performed as above with the exception that no attempt was made to count the total number of colonies as had been done in the previous cases with *S. aureus* and *E. typhosa*. Instead, the plates were classified into two groups, according to the presence (+) or absence (—) of hemolysis. The results of this test are shown in Table III.

The *in vitro* test employed on *M. tuberculosis* was performed as explained previously for *S. aureus* and *E. typhosa*, with the exception of a slight change in inoculation of the medium. To one set of dilutions—1:10,000, 1:30,000, 1:50,000, 1:70,000, and 1:100,000—of each compound, 0.5 ml. of the treated positive tubercular sputum was added. After periods of one, ten, and sixty minutes, a standard 4-mm. loop of the organism compound mixture was streaked on sterile Petragnani's slants and incubated at 37° for six weeks. Results of these readings are recorded in Table III.

RESULTS

***In vitro* Results on *Staphylococcus aureus* 209.**—Table III includes all the results of the *in vitro* tests performed in the experiment. Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *S. aureus* (209) in a dilution of 1:2500 after a one-minute exposure and in a 1:5000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride shows antibacterial properties in a 1:2500 dilution after a one-minute exposure and in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride shows antibacterial properties in a 1:5000 dilution after a one-minute exposure and in a 1:10,000 dilution after a sixty-minute exposure. The two compounds, stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride and stilbestryl-bis (2-diethyl-amino-

propyl) ether (free base), show no evidence of possessing antibacterial properties in the five dilutions employed.

In vitro Results on *Eberthella typhosa*.—Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *E. typhosa* in a 1:5000 dilution after a ten-minute exposure and in a 1:20,000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride is antibacterial to this organism in a 1:5000 dilution after a one-minute exposure and in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride is antibacterial to this organism in a 1:2500 dilution after a sixty-minute exposure. Stilbestryl-bis [3(2 methylpiperidino) propyl] ether dihydrochloride is antibacterial in a 1:5000 dilution after a one-minute exposure and in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis (2-diethylaminopropyl) ether (free base) shows complete absence of antibacterial properties in the five dilutions employed.

In vitro Results on *Streptococcus pyogenes*.—Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *Strep. pyogenes* in a 1:10,000 dilution after a ten-minute exposure and in a 1:20,000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride is antibacterial in a 1:5000 dilution after a one-minute exposure and in a 1:20,000 dilution after a ten-minute exposure. Stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride is antibacterial in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride is antibacterial in a 1:10,000 dilution after a one-minute exposure and in a 1:20,000 dilution after a ten-minute exposure. Stilbestryl-bis (2-diethylaminopropyl) ether (free base) failed to show evidence of any antibacterial properties in the dilutions employed.

In vitro Results on *Mycobacterium tuberculosis* var. *hominis*.—Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *M. tuberculosis* var. *hominis* in a 1:10,000 dilution after a ten-minute exposure and in a 1:50,000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride is antibacterial in a 1:30,000 dilution after a one-minute exposure. Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride is antibacterial in a 1:10,000 dilution after a ten-minute exposure and in a 1:100,000 dilution after a sixty-minute exposure. Stilbestryl-bis (2-diethylaminopropyl) ether (free base) is antibacterial in a 1:10,000 dilution after a ten-minute exposure and in a 1:70,000 dilution after a sixty-minute exposure. The compound, stilbestryl-bis [2(4-morpholino)ethyl] ether dihydrochloride, failed to show evidence of being antibacterial in the five dilutions employed.

The three basic ethers of 4,4′-dihydroxydiphenyl-

TABLE III.—RESULTS OF *in vitro* STUDIES OF THE SEVERAL STILBESTROL DERIVATIVES ON BACTERIA

Compound	Organism	Antibacterial Dil. in 1000's	Activity Time
Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride; *p*H, 6.3–6.9	*S. aureus* 209[a]	2.5	1
		5	60
	E. typhosa[b]	5	10
		20	60
	Strep. pyogenes	10	10
		20	60
	M. tuberculosis (var. *hominis*)	10	10
		50	60
Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride; *p*H, 6.3–7.1	*S. aureus* 209	2.5	1
		10	60
	E. typhosa	5	1
		10	60
	Strep. pyogenes	5	1
		20	10
	M. tuberculosis (var. *hominis*)	30	1
Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride; *p*H, 5.6–6.6	*S. aureus* 209	5	1
		10	60
	E. typhosa	5	1
		10	60
	Strep. pyogenes	10	1
		20	10
	M. tuberculosis (var. *hominis*)	10	10
		100	60
Stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride; *p*H, 5.6–6.6	*S. aureus* 209	....	None
	E. typhosa	2.5	60
	Strep. pyogenes	10	60
	M. tuberculosis (var. *hominis*)	...	None
Stilbestryl-bis (2-diethylaminopropyl) ether (free base); *p*H, 6.4–7.0	*S. aureus* 209	...	None
	E. typhosa	...	None
	Strep. pyogenes	...	None
	M. tuberculosis (var. *hominis*)	10	10
		70	60

[a] One ml. of broth contained 12 × 10^6 organisms.
[b] One ml. of broth contained 9 × 10^9 organisms.

sulfone, Table II, were inactive *in vitro* against *E. typhosa*, *S. aureus* (209), and *M. tuberculosis* var. *hominis*.

IN VIVO TESTS

Three groups of ten guinea pigs each were numbered one to thirty inclusive and assigned to separate cages; group A—experimental (1–10); group B—drug control (11–20), and group C—organism control (21–30).

On the first day of the tests the weights of all animals were determined and recorded. The ten "experimental animals" and the ten "organism control animals" were injected subcutaneously with 0.5 ml. of a treated positive tubercular sputum. All of the "experimental animals" and all of the "drug control animals" were given their previously assigned doses of the stilbestrol derivatives intraperitoneally. These twenty animals continued to receive their respective doses of the stilbestrol derivatives in the same manner every forty-eight hours for a period of seven weeks. The weights of all animals were checked and recorded every seven days. When an animal died before the termination of the seven-week period, it was weighed and autopsied, and smears were taken of all organs which showed indications of tubercle lesions. The smears were stained by the Ziehl-Neelsen technique and examined for *M. tuberculosis*. At the end of the seven-week period all animals that had not died were weighed, killed, and autopsied, and smears were made of all organs which showed indication of tubercle lesions. These smears were also stained by means of the Ziehl-Neelsen method and examined for *M. tuberculosis*. Results of the *in vivo* studies are given in Table IV.

The "experimental animals," which received 0.5 ml. of treated positive sputum on the first day of the test and forty-eight-hour intraperitoneal injections of the stilbestrol derivatives for a period of seven weeks, showed on autopsy and staining, tubercle bacilli at all sites of injection of the organism and at least two organs. Animals numbered 6, 7, and 9 died on the 18th, 6th, and 26th days, respectively. All of the ten animals of the "drug control group," which received forty-eight-hour intraperitoneal injections of the stilbestrol derivatives for the seven-week period, survived. With the exception of animals numbered 13 and 14 the change in body weight over the seven-week period was negligible. Animals numbered 13 and 14, which received the 150 and 300 μg. doses, respectively, of stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride, showed

TABLE IV.—RESULTS OF THE *in vivo* STUDIES WITH M. TUBERCULOSIS VAR. HOMINIS[a]

Compound[b]	Animal Number	Dosage Every 48 Hrs. in μg.	Initial Wt., in Gm.	Wt. in Gm. at Autopsy	Day of Death and Autopsy	Location of Org. at Autopsy[c]	Wt. in Gm. on 49th Day of Test
I	1	150	570	427	49th	A, B, C, D, E	
	2	300	670	580	49th	A, B, C, D, E	
II	3	150	720	540	49th	A, D, E	
	4	300	720	617	49th	A, B, D	
III	5	150	520	360	49th	A, C, D	
	6	300	550	420	18th	A, B, D	
IV	7	150	600	450	6th	A, B, D	
	8	300	700	580	49th	A, B, C, D, E	
V	9	150	450	340	26th	A, B, D, F	
	10	300	500	407	49th	A, C, D, E	
I	11	150	450	...	...	...	440
	12	300	450	...	...	...	445
II	13	150	420	...	...	...	340
	14	300	450	...	...	...	360
III	15	150	450	...	...	...	497
	16	300	500	...	...	...	547
IV	17	150	400	...	...	...	417
	18	300	450	...	...	...	490
V	19	150	400	...	...	...	392
	20	300	520	...	...	...	542
	21	...	420	240	41st	A, D	
	22	...	400	360	49th	A, B, E	
	23	...	450	292	49th	A, B, D, E	
	24	...	370	285	49th	A, B, D, E	
	25	...	420	340	49th	A, B, D, E	
	26	...	400	392	49th	A, B, D, E	
	27	...	440	367	49th	A, B, D, E	
	28	...	520	480	49th	A, B, E	
	29	...	450	390	45th	A, E, F	
	30	...	450	270	33rd	A, B	

[a] Animals 1–10 represent the guinea pigs of the Experimental Group injected subcutaneously on the first day of the experiment with 0.5 ml. of positive treated tubercular sputum. Animals 11–20 Drug Control Group. Animals 21–30 represent the guinea pigs of the Organism Control Group injected subcutaneously on the first day of the experiment with 0.5 ml. of positive treated tubercular sputum.

[b] I [illegible] dihydrochloride.
II [illegible] dihydrochloride.
III [illegible] dihydrochloride.
IV—Stilbestryl-bis (3(2-methylpiperidino) propyl) ether dihydrochloride.
V—Stilbestryl-bis (2-diethylaminopropyl) ether (free base).

[c] A—Site of injection of organisms, B—liver, C—spleen, D—lung, E—lymph gland, F—intestine.

a loss of body weight of 80 Gm. in the first case and 90 Gm. in the second.

All animals of the "organism control group," which received 0.5 ml. of treated positive sputum on the first day of the experiment, showed on autopsy and staining *M. tuberculosis* at all sites of injection of the organism and in at least one organ. A marked loss of weight was observed in these animals over the seven-week period. Animals numbered 21, 29, and 30 died on the 41st, 45th, and 33rd days, respectively.

SUMMARY

1. The preparation of a series of substituted aminoalkyl ethers of diethylstilbestrol and 4,4′-dihydroxydiphenyl sulfone is described.

2. Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride was the most active of the group *in vitro* using *S. aureus*, *Strep. pyogenes*, and *M. tuberculosis* var. *hominis*. The 2-diethylaminoethyl ether of diethylstilbestrol was most active against *E. typhosa*. The basic ethers of 4,4′-dihydroxydiphenyl sulfone showed no activity in the *in vitro* tests.

3. The basic ethers of diethylstilbestrol were shown to be inactive when tested therapeutically in guinea pigs infected with *M. tuberculosis*. All uninfected animals which received forty-eight-hour injections of the basic ethers of diethylstilbestrol survived the seven-week observation period.

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Studies on the Pharmacology of *Euphorbia pilulifera**,†

By LLOYD W. HAZLETON and REBECCA C. HELLERMAN

Pharmacologic, antianaphylactic, and antihistaminic properties of *Euphorbia pilulifera* were investigated. Dealcoholized fluidextract of euphorbia was used except where otherwise noted. Oral doses of 30 cc./Kg. to mice and 15 cc./Kg. to guinea pigs were not acutely toxic. Rats fed 1.0 per cent and 5.0 per cent powdered euphorbia in the basic laboratory diet for ninety-seven days gained weight normally and showed no gross pathology on autopsy. Blood pressure and respiration studies in dogs showed that euphorbia administered intravenously resulted in a marked but fleeting vasodepression with little effect on respiration. Guinea pigs actively sensitized to egg white were protected from subsequent lethal anaphylactic shock by oral administration of euphorbia about one hour prior to injection of egg white. Euphorbia antagonized the action of histamine on isolated guinea-pig intestine.

INTRODUCTION

THE PLANT *Euphorbia pilulifera* is one of some 2500 members of the euphorbiaceæ family. In the National Formulary, Seventh Edition (1), *Euphorbia pilulifera* was designated as pill-bearing spurge but was deleted from the Eighth Edition. The drug of commerce consists of the dried plant. Many members of the euphorbiaceæ family are described as being relatively toxic and containing resins or latex and it is therefore important to distinguish the pilulifera from other members of its family in considering its pharmacology and therapeutic uses.

A review of textbook material (2–4) reflects the traditional use of *Euphorbia pilulifera* in the treatment of such conditions as asthma, hay fever, bronchitis, and other respiratory conditions. The synonyms of the plant, as well as of its preparations, further substantiate the observations that the drug has long been associated with the treatment of these disorders. In the latter part of the Nineteenth Century *Euphorbia pilulifera* (5, 6) received considerable clinical attention for its use in the treatment of asthma and a review of the literature will indicate that from the clinical standpoint it was considered to be quite effective. This popularity is reflected in the compound elixir of euphorbia, National Formulary, Seventh Edition (1), which carries the synonym of Hare's Elixir. In commerce this preparation was known as Hare's Anti-Asthmatic

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Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
[illegible] de of a grant from Templetons, [illegible] and Templetons, Incorporated, [illegible] f this research.

Elixir and is prepared from the fluidextract of *Euphorbia pilulifera*, also official in National Formulary, Seventh Edition.

Subsequent to the period referred to above little, if any, scientific work was conducted on *Euphorbia pilulifera*, but during this time the techniques and methodology of investigating allergic and asthmatic conditions underwent tremendous strides. Among the significant developments was the elucidation of the possible role of histamine as a factor in anaphylactic shock and allergies. Significant progress was made in the experimental studies of shock, which came to be known as foreign protein reaction and is typified by the lethal effects of injected egg white in guinea pigs following prior sensitization to this material. More recently exhaustive studies have been conducted on two groups of synthetic drugs, which are referred to generally as antihistaminic agents and antispasmodics.

In view of the long history of use, the earlier significant clinical studies and the lack of recent investigations taking advantage of the improved techniques available, it seemed desirable to re-evaluate the pharmacology of euphorbia on an experimental basis. In the report to follow, any reference to euphorbia designates *Euphorbia pilulifera*.

PREPARATIONS USED

The following preparations were used during the course of the studies:

1. Finely ground *Euphorbia pilulifera*, the pharmacognostic authenticity of which was established through the cooperation of Dr. Heber W. Youngken. This material *per se* was used in the subacute feeding experiments.

2. From the powdered euphorbia fluidextracts were prepared according to the National Formulary, Seventh Edition, using Process A, with diluted alcohol as the menstruum. Due to the alcohol content the fluidextract was not used directly in any of the studies.

3. From the fluidextract a dealcoholized fluidextract was prepared by evaporating a measured quantity of the fluidextract over a water bath to a syrupy consistency and making up to original volume with distilled water. This material was used for the determination of oral toxicity, for the investigation of smooth muscle activity and antiallergic properties, and is referred to in the text as dealcoholized fluidextract.

4. To clarify the dealcoholized fluidextract and to remove insoluble vegetable extractives, this material was filtered through Lloyd Reagent (a purified aluminum silicate). This material was used for the study of the pharmacological properties following intravenous administration and in some of the smooth muscle experiments, and is referred to as partially purified.

5. To investigate in a preliminary manner the stability of the active principles, portions of the above clarified solution were placed in containers immersed in boiling water for fifteen minutes, and checked for potency of smooth muscle activity.

6. Since it has been reported (2) that *Euphorbia pilulifera* might contain an unstable glucoside, the clarified solution was treated with *N/10* hydrochloric acid, 1 cc. to 5 cc. of solution, maintained at the temperature of boiling water for fifteen minutes, then checked for potency of smooth muscle activity.

EXPERIMENTAL

Acute Toxicity.—Dealcoholized fluidextract of euphorbia was given to mice by oral administration (Table I). The dose was limited by the large volume, 75 cc./Kg. being as large as was practical to administer. There was very little effect except for slight depression and catharsis at the higher doses.

TABLE I.—ACUTE TOXICITY FOR MICE OF ORALLY ADMINISTERED DEALOCHOLIZED FLUIDEXTRACT OF EUPHORBIA

Dose, Cc./Kg.	Number of Mice	Living	Dead
5	2	2	0
10	2	2	0
20	2	2	0
30	10	10	0
60	10	8	2
75	10	10	0

Partially purified dealcoholized fluidextract of euphorbia, preparation 4, was given intravenously to mice (Table II). The injections were followed by very rapid respiration, marked vasodilation visible in the ears and tails, and occasional convulsions. One of the survivors at 10 cc./Kg. had hemorrhages into the eyeballs and sockets but no other gross pathology was observed. The LD_{50} is 7.4 cc./Kg. with an f of 1.28 (7).

TABLE II.—ACUTE TOXICITY FOR MICE OF INTRAVENOUSLY ADMINISTERED DEALCOHOLIZED FLUIDEXTRACT OF EUPHORBIA (FILTERED THROUGH LLOYD REAGENT)

Dose, Cc./Kg.	Number of Mice	Living	Dead[a]
5.0	12	9	3
7.5	10	5	5
10.0	14	4	10

[a] LD_{50} = 7.4 (f = 1.28).

Intravenous administration of lethal doses to rabbits resulted in marked respiratory stimulation followed by slowing and cessation while the heart was still beating.

Prior to tests for protective action against anaphylactic shock, oral doses of 0.5 cc./Kg. to 15 cc./Kg. were given to guinea pigs with no apparent toxicity.

Chronic Toxicity.—Three groups of 10 male albino rats each were used in chronic feeding experiments. The control group received laboratory diet only, the second group received laboratory diet containing 1% added euphorbia, while the third group received laboratory diet containing 5% of euphorbia. Each rat was individually housed and

had free access to food and water. Body weight and food consumption were determined weekly. One rat in the 5% level died after eighty-eight days from respiratory and middle ear infection. After ninety-seven days all remaining rats had gained weight normally (Table III). Following this period the rats were injected with 1 cc. egg white intraperitoneally (8). Euphorbia had no effect on the nonlethal hypersensitivity normally observed in the rat. The rats showed no gross pathology at autopsy.

Repetition of these experiments was made during March, April, and May, when the diet consisted primarily of dry pellets with only supplemental greens. A second lot of fluidextract of euphorbia prepared from the original lot of crude drug was used for these experiments. In contrast to the uniformity obtained in the first series, this second series showed marked irregularity, both in controls and treated groups. Of 16 animals, 3 proved to be totally immune to the administration of 3 or more

TABLE III.—SUMMARY OF FOOD CONSUMPTION FOR RATS ON SUBACUTE FEEDING OF EUPHORBIA FOR A PERIOD OF 97 DAYS[a]

Group	No. of Rats Start	Finish	Av. Weight Start	Finish	No. of Rat Days	Percentage of Theoretical	Total Food and Euphorbia Consumed, Gm.	Food Consumed: Total, Gm.	Food Consumed: Av./Rat/Day, Gm.	Euphorbia Consumed: Total, Gm.	Euphorbia Consumed: Av./Rat/Day, Gm.	Euphorbia Consumed: Av. Total/Rat, Gm.
Control	10	10	105	336	970	100	19,463	19,463	20.1	....	..	....
1% Euphorbia	10	10	106	329	970	100	20,228	20,025.72	20.6	202.28	0.21	20.37
5% Euphorbia	10	9	105	320	961	97.1	21,035	19,983.25	20.8	1051.7	1.09	105.73

[a] Two animals from the control group, 2 from the 1%, and 3 from the 5% levels died of respiratory infections during the first ten days. These were replaced with rats of the same stock and no allowance for this replacement is made in the total.

Guinea-Pig Egg White Sensitization.—Active sensitization was induced in guinea pigs (9) by intraperitoneal injection of 5 cc. of egg white, 25% in physiologically normal saline. After two or more weeks the injection of 0.75 cc. undiluted egg white resulted in death from anaphylactic shock. Death was preceded by coma and severe dyspnea, accompanied by audible gasping and convulsive movements.

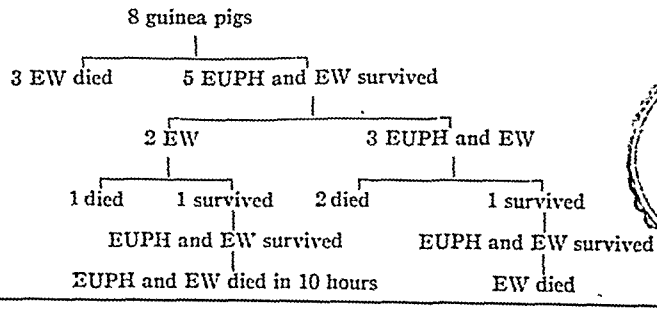
In an initial series of experiments, conducted during August and September, when the diet was primarily greens with pelleted food available, the oral administration of 15 cc./Kg. of dealcoholized fluidextract of euphorbia to sensitized guinea pigs one hour prior to intraperitoneal injection of egg white exhibited a marked antianaphylactic effect (Table IV). On the first test all three controls died in an average time of sixty-four minutes while five treated with euphorbia survived. Subsequent tests indicate that one of these might have been relatively immune to egg white, since it survived one dose unprotected. The most marked symptom of relief, aside from survival, was ease of respiration with total absence of audible gasping. Most of the animals became comatose for at least short intervals.

injections of egg white. The average time of death for 5 control animals was twenty-two minutes, while the average time for 8 treated animals was twenty-eight and a half minutes. This difference in survival time is not significant ($P = 0.2$). It should be noted that the survival time of the control animals approximates only one-third of the survival time of the control animals in Table IV, while the incidence of total immunity is marked. The group thus exhibited both unusual susceptibility and resistance to anaphylaxis in comparison to the initial studies.

At present it is impossible to evaluate properly this second series of experiments, since a new fluidextract, a change of diet, a different season, and an apparent extreme variability in response to administered egg white are factors to be considered. It is planned to repeat these studies to eliminate season and diet as possible variables.

To evaluate further the influence of euphorbia on respiration during egg white shock, several large sensitized guinea pigs were anesthetized with sodium pentobarbital and the respiration recorded by air tambour from the trachea. One guinea pig which served as a control had normal anesthetic respiration

TABLE IV.—INFLUENCE OF ORALLY ADMINISTERED DEALCOHOLIZED FLUIDEXTRACT OF EUPHORBIA ON LETHAL EGG WHITE ALLERGY IN ACTIVELY SENSITIZED GUINEA PIGS. EW = EGG WHITE; EUPH = EUPHORBIA GIVEN 1 HOUR IN ADVANCE OF EW



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prior to the intraperitoneal injection of 0.75 cc. of egg white, after which the respiration became very poor and fluid formed in the tracheal canula. The respiration became slower and more shallow and ceased one-half hour after the injection of egg white. A second control animal had normal respiration before the injection of 0.75 cc. of egg white (Fig. 1).

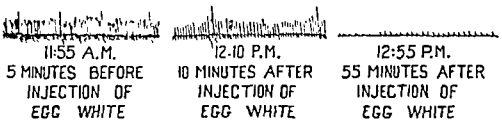

Fig. 1.—Respiratory tracings taken at intervals during egg white shock in unprotected guinea pig.

The respiration became slower and shallow fifteen minutes after the injection of the egg white. In one hour there was fluid in the tracheal canula, the respiration was very slow and shallow, and fifteen minutes later it stopped. A third guinea pig showing normal respiration was given 0.10 cc. of partially purified dealcoholized fluidextract of euphorbia intravenously. Following is an outline of the protocol describing the events shown in Fig. 2.

2:10—partially purified dealcoholized fluidextract of euphorbia, 0.10 cc. intravenously. Respiration more rapid.

2:15—0.75 cc. egg white intraperitoneally.

2:23—Respiration more shallow.

3:00—Respiration very shallow with some deep inspirations. 0.10 cc. euphorbia intravenously resulted in rapid respiration for about one minute.

3:33—0.10 cc. euphorbia intravenously. Respiration rapid for about one minute following injection, then it improved, was more regular, and there was an absence of the deep inspirations.

3:55—0.10 cc. euphorbia intravenously. Respiration rapid for about one minute. Respiration improved, looked similar to tracing before injection of egg white.

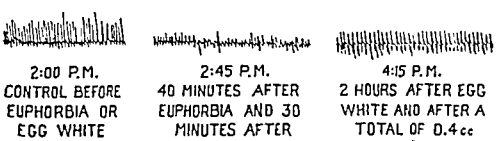

Fig. 2.—Respiratory tracings taken at intervals during egg white shock in guinea pig protected by intravenous injections of euphorbia.

No more euphorbia was given and no more records were made, but the guinea pig lived until about 8:00 o'clock, which was almost six hours after the injection of egg white. During the entire course of the experiment there was no fluid in the tracheal canula as there had been in the controls. A fourth guinea pig was set up in the same manner. Respiration was normal when 0.75 cc. egg white was given intraperitoneally. Respiration became very shallow and irregular. The tracheal canula became filled with fluid. Thirty-seven minutes after the egg white, 0.075 cc. partially purified euphorbia was given intravenously. This dose had very little effect and 0.25 cc. partially purified euphorbia was given intravenously fifty minutes after the egg white. The animal died immediately after this dose, with no evidence of relief. Whether death was due to the large dose of euphorbia *per se* or was influenced by the advanced condition of shock could not be determined. It is interesting that the average survival time for this animal and the two controls, under anesthesia, was fifty-five minutes.

Blood Pressure and Respiration: Dogs.—In dogs under barbiturate anesthesia the intravenous injection of the partially purified fluidextract of euphorbia produces a characteristic vascular and respiratory sequence. The blood-pressure response is biphasic with an initial rise followed by a more pronounced fall. The pressor phase appears to be subject to tachyphylaxis since it may disappear after several doses. Figure 3 presents alternate injections of histamine and euphorbia, clearly indicating lack of antihistaminic action on the blood-pressure response. In general the depressor action of euphorbia resembles that of histamine, although the recovery in Fig. 3 (*d*) is more rapid than usual. The duration of action is brief and there is no evidence of accumulation.

Respiration was recorded by means of a Pfeiffer canula (10) using flutter valves. This maintains a constant minimum resistance to inhalation and expiration and therefore adds significance to these phases of the tracings. The respiratory effects appear to be primarily compensatory, and again the resemblance between histamine and euphorbia (Figs. 3 (*a*) and (*b*)) is marked. Following the initial dose of euphorbia, however, the nature of the respiratory response is markedly changed. While this change is apparent in the rate and duration it is more characteristically seen in the inspiratory phase as recorded by the air tambour from the trachea, which remains almost normal. Larger doses of euphorbia produce marked respiratory stimulation followed by apnea.

Euphorbia has no significant effect on the hemodynamic actions of epinephrine or acetylcholine and is not antagonized by doses of atropine which paralyze the parasympathetic receptor mechanism.

The following experiments were designed to determine whether the vasodepressor phase might be due to histamine or comparable substances in the plant extractive. A solution of histamine diphosphate was adjusted to *p*H 5.2 by the addition of potassium phosphate monobasic to bring it within the *p*H range of the partially purified fluidextract (*p*H 4.5). A portion of this solution was then filtered through Lloyd Reagent. In an anesthetized dog the buffered solution exhibited normal histamine activity while the Lloyd Reagent treated portion was totally devoid of activity. While this indicates that any histamine would have been removed by filtering the fluidextract through Lloyd Reagent, the dog was injected with 3 mg./Kg. of Tripelennamine hydrochloride (Pyrabenzamine HCl) intravenously. This dose almost completely antagonized the histamine but had no appreciable effect on the vasodepressor action of euphorbia.

Additional evidence of myotropic activity by euphorbia was obtained from two dogs severely

poisoned by O,O-diethyl-O-*p*-nitrophenyl thiophosphate (parathion), a cholinesterase inhibitor (11–13). Atropine administered intravenously to these dogs effectively antagonized the vagal stimulation and temporarily increased the respiratory exchange as recorded by the tambour. As respiratory exchange again decreased the intravenous administration of partially purified euphorbia produced its characteristic biphasic vascular response and increased respiratory exchange. Improvement occurred in both the inspiratory and expiratory phases, and with both atropine and euphorbia can probably be attributed to bronchial dilation, neurotropic and myotropic, respectively, since there is no change in rate.

Isolated Guinea-Pig Ileum.—Conventional techniques were employed in the study of isolated seg-

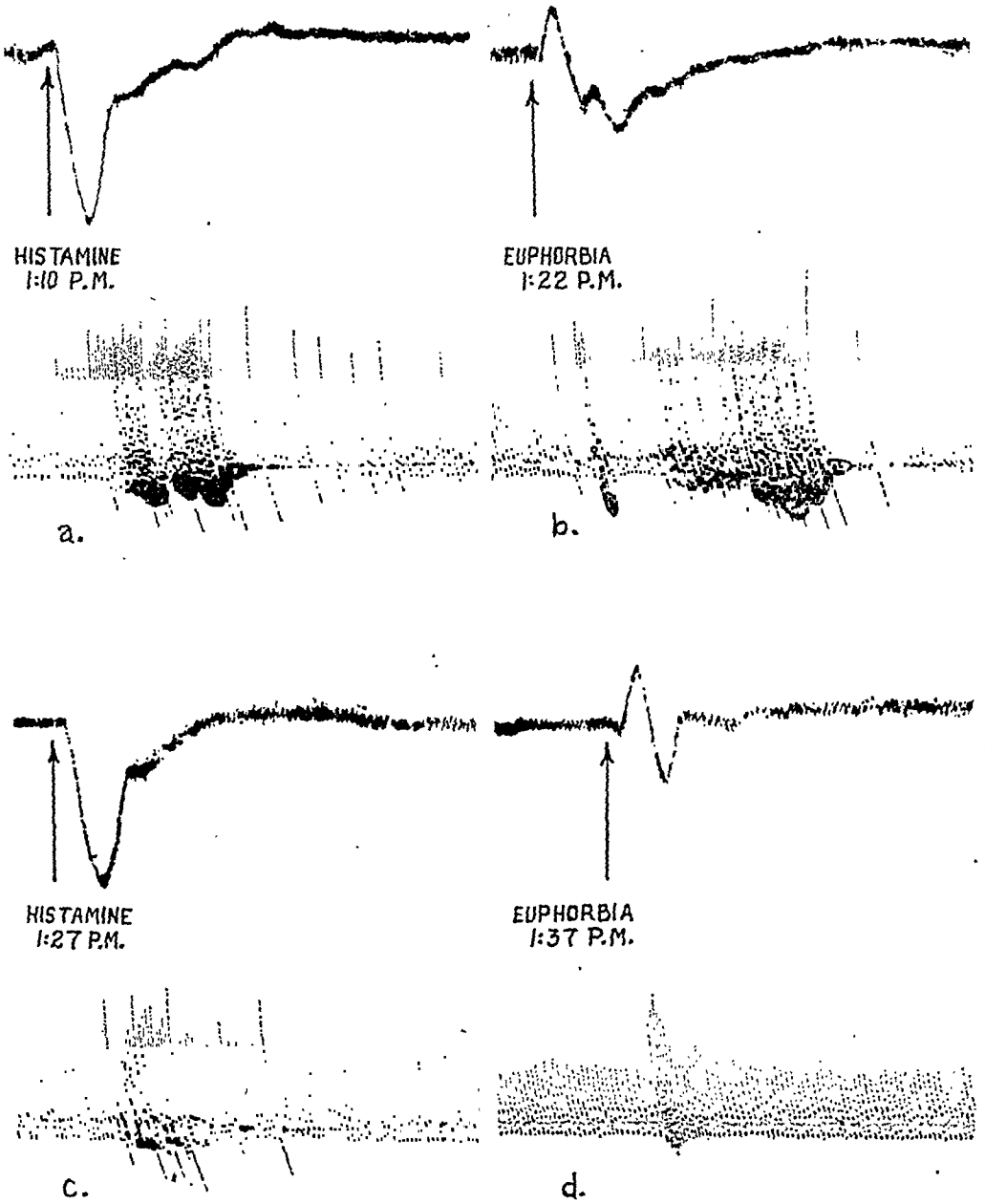

Fig. 3.—Alternate intravenous injections of histamine and euphorbia in dog weighing 10 Kg. under barbiturate anesthesia. Histamine was injected as the phosphate, 0.01 mg./Kg. Euphorbia was injected as the partially purified dealcoholized fluidextract, 0.1 cc./Kg. The top tracing is the blood pressure recorded from the carotid artery by mercury manometer. The bottom tracing is the respiration recorded from the trachea by air tambour.

ments of guinea-pig small intestine. The segments were placed in a chamber of 100-cc. capacity, stirred, and oxygenated by small bubbles of oxygen. The surrounding bath was maintained at 37° C. The lever was maintained at a ratio writing double the muscle excursion. Locke's solution was used exclusively in the investigations to be reported. Quantities of drugs quoted are those added to the chamber and therefore do not represent the final dilution in contact with the muscle.

In the early experiments the dealcholized fluidextract was filtered through gauze to remove the larger precipitate particles. Typical reactions of this preparation are illustrated in Figs. 4 (*a*), (*b*), and (*c*), all consecutive from the same intestinal segment. After standardization of the histamine dose (Fig. 4 (*a*)) the bath was washed and 1 cc. of the euphorbia added (Fig. 4 (*b*)). The sharp initial spike reaction appears to be characteristic of the drug as represented by the fluidextract. Repetition of the histamine now is without effect on the muscle. That the euphorbia is removable by washing is illustrated in Fig. 4 (*c*), where the response to the original dose of histamine is comparable. Complete relaxation

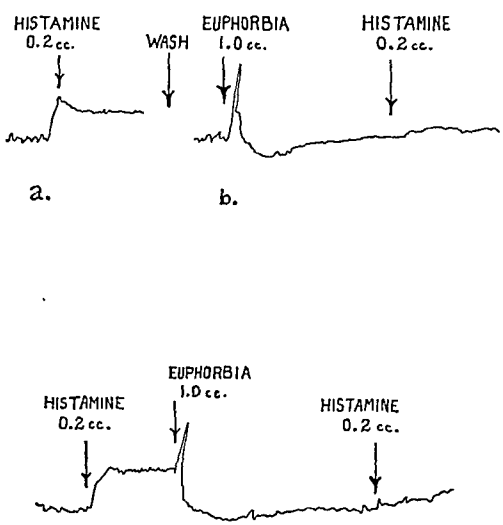

Fig. 4.—Relaxation and prevention of histamine (0.01 mg./cc.) contraction of guinea-pig ileum by euphorbia. (*a*, *b*, and *c* are consecutive tracings.)

of the histamine contraction, preceded by the characteristic spike, is achieved by the addition of euphorbia. A second dose of histamine at this point does not initiate a contraction. It should be noted that euphorbia did not relax the muscle appreciably below the normal baseline nor interfere with normal rhythmic activity. Dealcoholized fluidextracts of quassia, senna, and ergot were similarly tested for comparison. Quassia and senna exert no appreciable effect on the muscle and do not interfere with histamine contraction and its relaxation by euphorbia. On the other hand the ergot action is similar to euphorbia, producing a spike contraction and histamine antagonism without stopping rhythmic activity.

The activity of the total fluidextract of euphorbia was not diminished by filtration through Lloyd Reagent although there appears to be a marked diminution of the spike phase. Figures illustrating this on the ileum are omitted since the activity is retained after heating this preparation with acid (preparation 6) illustrated in Figs. 5 (*a*) and (*b*). Pertinent factors to be noted in Figs. 5 (*a*) and (*b*) are the ability of this preparation both to relax and prevent contraction from a relatively large dose of histamine in an active segment, the reduction of the spike phase, and the continuation of normal rhythmic activity following relaxation by euphorbia. In Fig. 5 (*c*) is illustrated the relaxation of a strong acetylcholine contraction, with no spike phase, by the partially purified product.

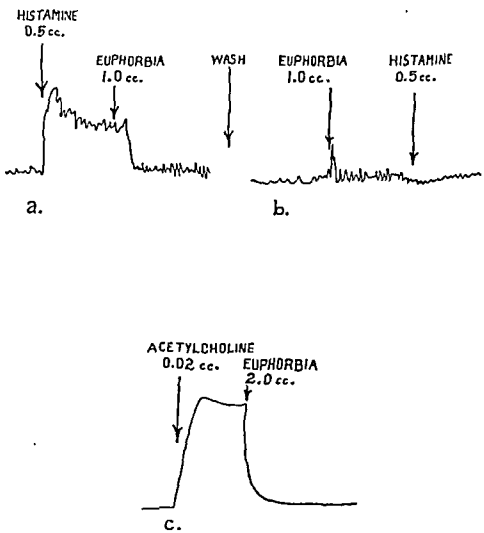

Fig. 5.—(*a* and *b*) Relaxation and prevention of histamine (0.01 mg./cc.) contraction of guinea-pig ileum by euphorbia filtered through Lloyd Reagent and heated with HCl in boiling water. (*c*) Relaxation of acetylcholine (0.1 mg./cc.) contraction by euphorbia filtered through Lloyd Reagent.

Preliminary experiments on differential extraction, not sufficiently complete to merit detailed discussion, indicate that (1) ether extraction of the dealcoholized fluidextract does not remove the active principle(s), the aqueous residue retaining most of the depressant and spike properties, (2) the ether portion, after removal of the ether, shows primarily stimulant properties, (3) an aqueous extract of euphorbia, representing 1 Gm./cc., contains about one-half the antihistaminic activity of the fluidextract, with proportional spike tendency, (4) an alcohol (95%) extract of the moist marc from the above aqueous extract, with the alcohol removed and replaced by water to represent 1 Gm. of the original drug per cc., contains almost entirely the stimulant or spike phase. The action closely resembles histamine, will reinforce a histamine contraction, and is antagonized by the dealcoholized fluidextract.

DISCUSSION AND SUMMARY

The empirical use and available literature on euphorbia suggest pharmacological investigations directed at an antihistaminic activity. Certain of the properties reported herein, such as the prevention or relaxation of histamine contractions of the guinea-pig ileum and a moderate degree of protection against egg white shock are suggestive of such action.

Certain other properties including relaxation of acetylcholine contraction, relaxation of bronchial constriction induced by chemicals other than histamine, and failure to counteract the vascular effects of injected histamine suggest that the action is not specifically antihistaminic. Supporting evidence is the absence of protection against egg white induced coma in guinea pigs and peripheral edema in rats.

Since euphorbia exerts no effect on the hemodynamic actions of epinephrine or acetylcholine and is not antagonized by atropine it may be assumed that the vascular actions are independent of the autonomic nervous system. The action is biphasic with only slight evidence of tachyphylaxis in the initial pressor phase and no evidence of accumulation.

The uniformity of these responses indicates a direct depressant action on smooth muscle rather than a specific antihistaminic or antiallergic activity. It is apparent after either oral or parenteral administration. This action is typically antispasmodic since doses which relax induced spasms of the ileum or bronchi have little effect on the normal tissue and do not interfere with normal rhythmic contractions of the ileum.

During the course of the investigations it became apparent that at least two active principles are present in *Euphorbia pilulifera*. The first of these caused a spike phase in guinea-pig ileum and was removed by filtration through Lloyd Reagent. In a review on histamine Gaddum (14) reports that Fuller's earth, magnesium trisilicate, and, under certain conditions, charcoal are capable of absorbing histamine and outlines certain conditions to be met for biologically identifying this material in mixtures. Although it was demonstrated that Lloyd Reagent does absorb histamine in the *p*H range of fluidextract of euphorbia, this cannot be taken as positive proof of its identity in euphorbia. The spike-producing principle can be partitioned into ether from the dealcoholized fluidextract, or extracted from the moist marc after aqueous extraction of the crude drug. It is therefore soluble in both water and organic solvents, perhaps more so in the latter.

The second principle appears to be the one responsible for the predominantly relaxing action on the various smooth muscles studied. It is water soluble, is not absorbed by Lloyd Reagent nor hydrolyzed by a mineral acid at the temperature of boiling water. It is not extracted from solution by ether in the acid *p*H range nor from the water-exhausted marc by alcohol. In aqueous solution the principle is stable over long periods of time at room temperature.

The acute and chronic toxicity of *Euphorbia pilulifera* is of an order which permits adequate dosage for pharmacological investigation with a substantial margin of safety. The mechanism of lethal action is not elucidated by the studies reported but it was observed that the principal symptoms of toxicity following intravenous injection are general depression and respiratory stimulation followed by depression and cessation. It is interesting to note that these observations coincide with those reported following oral administration to frogs, guinea pigs, and rabbits by Marsset in 1885 (6). This author also reported no observable direct effect on blood vessels, bronchi, alveoli, spinal cord, muscle (apparently skeletal), capillaries, or secretory glands. Within the limits of comparable techniques those observations are in general corroborated by the current investigations. The use of isolated tissue, intravenous administration, partial purification, histamine, and other pharmacological standards and the allergic phenomenon in the current studies serve to broaden appreciably the scope and elaborate these basic observations.

CONCLUSIONS

1. Fluidextract of *Euphorbia pilulifera* is relatively nontoxic following oral administration to mice.

2. The intravenous LD_{50} of the fluidextract, after filtering through Lloyd Reagent, is 7.4 cc./Kg. in mice.

3. The whole drug in levels up to 5% of the diet produced no toxicity in rats within ninety-seven days.

4. After oral administration the fluidextract exerts a prophylactic action against lethal egg white shock in guinea pigs.

5. Euphorbia appears to contain at least two active principles, the properties of which are discussed.

6. The antispasmodic action on smooth muscle predominates, with evidence presented that this mechanism is directly myotropic.

7. Since the antispasmodic principle is not adsorbed by Lloyd Reagent nor hydrolyzed by weak mineral acid, euphorbia does not appear to be alkaloidal or glycosidal in nature.

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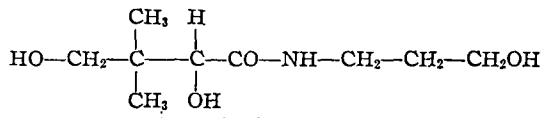
A Rapid Bioassay in Rats for Panthenol*

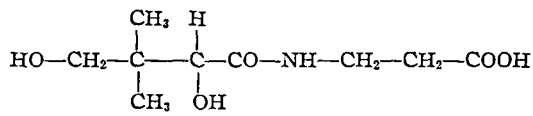
By L. DREKTER, R. DRUCKER, R. PANKOPF, J. SCHEINER, and S. H. RUBIN

An availability type of bioassay is presented for panthenol, the biologically active analog of pantothenic acid. The method is based upon the urinary excretion of pantothenic acid by the rat after dosage with panthenol. Pantothenic acid is determined by microbiological assay in the twenty-four hour urine. The specificity of the method has been demonstrated by comparison with rat curative and microbiological assays. The precision of the method, at suitable dosage levels, is of the order of 3 per cent in 8-rat assays.

In 1943 Pfaltz (1) showed that panthenol (I), the alcohol analog of pantothenic acid (II), is as effective a vitamin as the latter with regard to influence on growth and achromotrichia of black rats and alopecia in mice.



Panthenol (I) (mol. wt. = 205.3)



Pantothenic Acid (II) (mol. wt. = 219.2)

Burlet (2) later demonstrated that panthenol is oxidized by the rat and by man to pantothenic acid, as judged by urinary excretion of the latter. Burlet further found that, under certain conditions of dosage, the availability of panthenol to the rat and to humans exceeds that of calcium pantothenate, an observation which we (3) have recently confirmed and extended in humans.

Since recent trends (4, 5) in vitamin methodology have stressed the desirability of bioassays which measure *availability* to mammals, and because of the likelihood that panthenol is active by virtue of conversion to pantothenic acid, it was deemed desirable to evolve an assay method of the availability type. The method presented here depends upon measurement of the urinary pantothenic acid of rats after dosage with panthenol.

EXPERIMENTAL

Handling of Rats.—Male, albino rats weighing 150 Gm. or more were housed in pairs in metabolism cages. Improved cages, designed to give better separation of urine and feces, were used and will be described elsewhere (6). The rats were given distilled water and stock diet ("Friskie Dog Chow") *ad libitum*, the latter being ground and then wetted down with equal amounts of water to minimize spillage. After dosage with the panthenol solution, as described below, urines were collected for twenty-four hours in small, amber bottles under toluene. At the end of the collection period, the rats were removed from the cages, the lower screen containing the feces was removed, and the entire cage washed down (with the aid of a rubber policeman) with enough distilled water to bring the urine and washings to 1 liter.

Microbiological Assay.—Further dilutions were made as required for microbiological assay of the urinary pantothenic acid by the method of Skeggs and Wright (7), employing *Lactobacillus arabinosus*. The turbidity of the bacterial growth was measured in an Evelyn colorimeter after sixteen hours' incubation at 37° in a forced-draft incubator room.

It is not essential, though desirable, to analyze the urine from each cage separately. In the experiments described, up to 10 cages were used per sample and the urine from each was analyzed individually to provide data for statistical evaluation, presented below.

Basal Excretion.—In our panthenol studies on human subjects (3), it was the practice, as is commonly done (4, 5) in availability assays on humans, to determine the basal excretion of the vitamin prior to dosage. The necessity for such repetitive determination is contingent upon the relative magnitude of basal and dose and the variations in the two in relation to the desired precision of assay. On the basis of the data shown in Tables I and II and Fig. 1, it seems feasible to use a "statistical" basal value, which, in the present experiments, averaged 0.13 ± S.E. 0.01 mg. in rats that had grown from *ca.* 150 to *ca.* 465 Gm. (Table I). As determined by the method of Ives and Strong (8), the Friskie Dog Chow provided 9 γ per Gm. of Ca pantothenate; since these rats consumed 12–17 Gm. of the chow daily, an intake of *ca.* 0.1–0.15 mg. of pantothenic acid is indicated. The pantothenic acid requirement of rats of the age group used has been variously put at 0.025 mg. (9) to 0.1 mg. (10). Equating these estimates of intake, utilization, and urinary excretion leads to the conclusion that the basal excretions found are within a plausible range.

* Received June 7, 1948, from Nutrition Laboratories, Hoffmann-La Roche Inc., Nutley, N. J.

TABLE I.—LACK OF EFFECT OF REPEATED DOSAGE[a] WITH PANTHENOL UPON BASAL URINARY EXCRETION OF PANTOTHENIC ACID

No. of Doses Received[b]	Weeks Used	No. of Rats	Weight, Gm.	Urinary Ca Pantothenate, Mg./Day Average	Range	Standard Error
0	0	54	150–180	0.13	0.08–0.17	0.01
5	5	60	275–320	0.13	0.08–0.19	0.03
18	18	20	388–465	0.15	0.12–0.17	0.01

[a] At intervals of one week.
[b] From 1 to 5 mg. of panthenol per dose by intramuscular injection.

It is further demonstrated in Table I that repeated dosage with panthenol over eighteen weeks at intervals of one week had no significant effect on the basal excretion. The interval of one week was chosen on the basis of control experiments in which it was found that the urinary excretion returns to the basal value within a week after dosage by injection or per os. Table II details a typical experiment, in which the excretion of pantothenic acid returned to basal levels by the fourth day after injection or feeding of a large dose of panthenol, 3.45 mg.

TABLE II.—DAILY URINARY EXCRETION OF PANTOTHENIC ACID AFTER A SINGLE LARGE DOSE OF PANTHENOL (3.45 MG.)[a]

Days after Dosage	Urinary Ca Pantothenate: Oral Dosage Mg.	Oral Dosage % of Dose	Intramuscular Injection Mg.	Intramuscular Injection % of Dose
Basal	0.12–0.17	3–4	0.12–0.17	3–4
1	2.36	59	2.56	64
2	0.38	9.5	0.34	8.5
3	0.24	6	0.22	5.5
4	0.21	5	0.19	5
5	0.17	4	0.17	4
6	0.14	3.5	0.18	4.5
7	0.14	3.5	0.15	4

[a] 3.45 mg. of panthenol ≈ 3.45/0.86 = 4.0 mg. of Ca pantothenate.

These considerations provide grounds for the use of a "statistical" basal value, which in our experiments has averaged 0.13 mg. per day.

Route of Administration of Test Dose.—The data in Table II further show that, after a test dose of 3.45 mg. of panthenol, the excretion of pantothenic acid is essentially the same whether the dose be given by mouth or by intramuscular injection. Table III demonstrates that this identity of response persists through lower levels of the standard dose-response curve given in Fig. 1 (v.i.). Both methods of dosage therefore provide the same sensitivity of response. However, as a matter of practical convenience, we prefer intramuscular dosage, since injection (into the sacrospinalis and multifidis muscles) is more easily done routinely than oral dosage.

Standard Dose-Response Curve.—In addition to the statistical basal value described above, it was deemed feasible similarly to construct a statistical (standard) dose-response curve, and so lessen the routine labor of this bioassay. Figure 1 summarizes 54 trials of panthenol by intramuscular injection in 108 rats of doses up to 5.1 mg. The curve is essentially linear at doses above 1 mg. The equation for this line, calculated by the method of least squares, is $y = -0.16 + 0.73x$; the standard error of the estimate ($Sy{\cdot}x$), is 0.17 mg. (11). For the individual means shown in Fig. 1, the coefficient of variation, $s/\bar{x}$, ranges from 1.4% at a dose of 5.1 mg. to 7% at a dose of 1 mg. This linear portion of the curve is therefore quite suitable for the assay. The curvilinear portion below 1 mg. is much less suitable and would entail considerably greater errors.

Specificity of Assay

Non-effect of Pantoic Acid and Pantoyl Lactone.—There is little reason to believe that these split products of panthenol would interfere in the sense that

TABLE III.—COMPARISON OF ORAL AND INTRAMUSCULAR ADMINISTRATION OF PANTHENOL IN RATS

Dose: Panthenol, Mg.	Dose: Ca Pantothenate Equivalent, Mg.	Urinary Ca Pantothenate: Oral, Mg./Day	Urinary Ca Pantothenate: Intramuscular, Mg./Day
3.45	4.0	2.36	2.56
1.72	2.0	1.25	1.21
0.43	0.50	0.40	0.35

these could be resynthesized in the rat to pantothenic acid, except for some data of Sarett (12) wherein, after feeding of pantoyl lactone or pantoic acid to humans, the pantothenic acid excretion increased from 3.5 to 6 mg. per day. In the present experiments (Table IV), the intramuscular injection of these split products, either alone or in combination with panthenol, had no effect upon the estima-

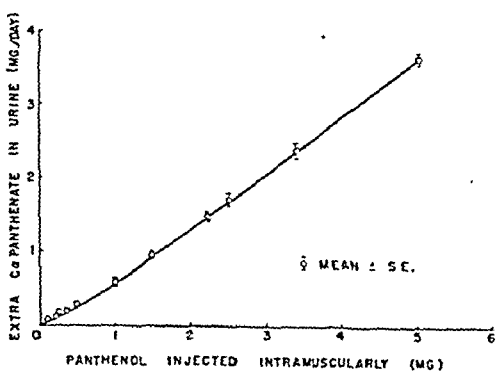


Fig. 1.—Relation between dosage with panthenol and urinary excretion of extra calcium pantothenate.

tion of the latter. Where panthenol was administered (expts. 3, 4, and 5, Table IV), the recoveries ranged from 92% to 96%.

TABLE IV.—NONEFFECT OF PANTOIC ACID AND PANTOYL LACTONE ON EXCRETION BIOASSAY OF PANTHENOL

No.	Injected	Panthenol Recovered Mg.[a]	%
1	3.5 mg. pantoic acid	0	..
2	3.3 mg. pantoyl lactone	0	..
3	2.5 mg. panthenol + 1.8 mg. pantoic acid	2.4	96
4	2.5 mg. panthenol + 1.7 mg. pantoyl lactone	2.3	92
5	2.5 mg. panthenol + 0.9 mg. pantoic acid + 0.9 mg. pantoyl lactone	2.4	96

[a] Corrected for basal excretion of 0.13 mg. per day.

Agreement with Curative Assays in Pantothenic-Deficient Rats.—The basic proof for specificity lies in comparisons with classical bioassays, wherein the vitamin deficiency is produced and cured. The chief limitation of such proof is the poorer precision of the curative bioassay. The comparison, nevertheless, provides a useful measure of specificity. For the curative assays, the diet used was essentially that of Henderson, *et al.* (10) with the following differences: 3% of cod-liver oil was substituted for 3% of the corn oil; biotin (2 μg.), *p*-aminobenzoic acid (0.25 mg.), inositol (0.5 mg.), and folic acid (2 μg.) were added to the vitamin supplement of Henderson, *et al.*, in the daily doses shown. The assays were conducted at two levels which, for the standard substance—calcium pantothenate—were 30 and 60 μg. per day; 10 rats were used at each level.

It was first necessary to ascertain the efficiency of panthenol in curing pantothenic deficiency in rats, relative to calcium pantothenate. Pfaltz (1) had previously demonstrated that these two substances have approximately equivalent activity in pantothenic deficiency. The present series of curative bioassays, based on growth responses, show that the activity is equivalent within the limits of error (v.i.) of the procedure, confirming Pfaltz' original observations. A typical set of response curves is detailed in Fig. 2.

The comparisons between the curative and excretion bioassays were carried out on ampuled solutions of panthenol which had been buffered in McIlvaine's phosphate-citrate buffer at graded *p*H from 3 to 8, preserved with methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate, and aged at 45° for periods up to sixty-three days. The results (Table V) expressed as a percentage value (excretion assay over curative assay) average 103% with a standard error of 2.7%, for the 15 samples tested. This agreement is eminently satisfactory, considering that two bioassays are involved. Since, as estimated by the method of Gridgeman (13), the standard error of a *single* curative bioassay was 8%, it is not surprising that the limits of the ratios observed range from 79% to 119%. The fact that the excretion method averaged 3% higher than the curative is not significant; *t* for the difference is 0.7; a *t* of 2.145 would denote significance at the 5% level.

Agreement with Microbiological Assays.—A further check on the specificity and precision of the excretion bioassay has been obtained by comparison

TABLE V.—COMPARISON BETWEEN CURATIVE AND EXCRETION METHODS FOR THE DETERMINATION OF PANTHENOL

Test Solution	Panthenol Found: Curative Assay, Mg./Cc.	Panthenol Found: Excretion Assay, Mg./Cc.	Panthenol Found: Excretion / Curative × 100, %
Initial solution	3.3	3.0	91
*p*H 3, 28 Days at 45°	2.3	2.5	109
*p*H 3, 42 Days at 45°	2.0	2.1	105
*p*H 3, 63 Days at 45°	1.8	2.0	111
*p*H 4, 28 Days at 45°	2.7	3.0	111
*p*H 4, 63 Days at 45°	3.0	2.8	93
*p*H 5, 28 Days at 45°	3.0	2.8	93
*p*H 5, 63 Days at 45°	2.9	2.9	100
*p*H 6, 28 Days at 45°	2.6	3.1	119
*p*H 6, 63 Days at 45°	2.9	2.9	100
*p*H 7, 28 Days at 45°	2.7	2.7	100
*p*H 7, 63 Days at 45°	2.3	2.6	113
*p*H 8, 28 Days at 45°	2.1	2.3	110
*p*H 8, 42 Days at 45°	2.4	1.9	79
*p*H 8, 63 Days at 45°	1.6	1.7	106
	Average = 103 ± (S. E.) 2.7		

with a microbiological assay method recently reported from this laboratory (14). Table VI gives typical comparative data by both methods for a vigorously aged multivitamin preparation. In order to cause partial decomposition, this solution was adjusted to *p*H 2 and heated at 45° for the

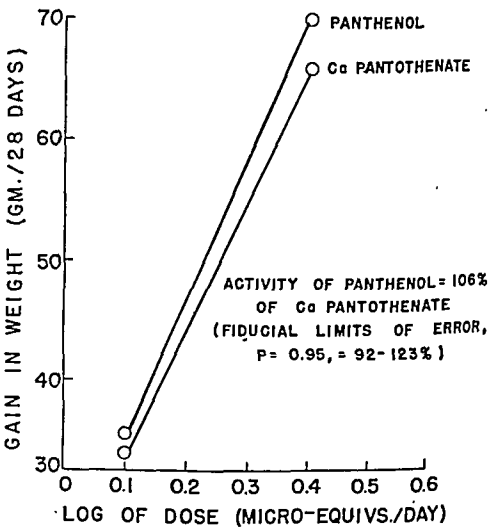


Fig. 2.—Relative activity in pantothenic-acid deficient rats.

periods shown in Table VI. This drastic treatment was necessary because of the slow rate of breakdown of panthenol under normal storage conditions. Satisfactory agreement between the two assay

methods was obtained for each solution, the average ratio being 102 ± 1.3% (S. E.). The deviation from 100% is not significant.

TABLE VI.—COMPARISON BETWEEN MICROBIOLOGICAL AND EXCRETION METHODS FOR THE DETERMINATION OF PANTHENOL

Test Solution,[a] Days at 45°	Panthenol Found: Microbiological Assay, Mg./Cc.	Panthenol Found: Excretion Bioassay, Mg./Cc.	Panthenol Found: Excretion Microbiol. × 100, %
0	3.8	3.7	97
1	3.5	3.6	103
2	3.3	3.4	103
3	3.0	3.0	100
5	2.7	2.8	104
8	2.5	2.5	100
12	2.2	2.2	100
16	2.0	2.0	100
20	1.8	2.0	111
	Average = 102 ± 1.3 (S. E.)		

[a] The test solution was a multivitamin preparation which was adjusted to pH 2 and aged at 45° in order to promote more rapid breakdown of panthenol, with a view, especially toward formation of pantoyl lactone.

Precision of the Excretion Bioassay.—As a measure of the working precision of the method, it has been useful to calculate the standard error of the excretion assays on these aged panthenol solutions. These were 4-cage assays, i.e., 8 rats per assay, at working levels of 1–3 mg. per rat. Urine from each cage was analyzed separately. For the 15 samples involved, the coefficient of variation, $s/\bar{x}$, ranged from 1% to 5.4%, with an average of 3.2%. Errors of similar magnitude were found when the method was applied to various pharmaceutical preparations, both solid and liquid. As noted above, the working errors are considerably greater at doses below 1 mg. It is therefore desirable that solutions for intramuscular injection contain at least 1 mg. of panthenol per cc.

Example.—The following example illustrates the procedure used in this laboratory to assay panthenol in a liquid multivitamin preparation with a claim of 5 mg. per cc. Into each of 8 rats, kept in pairs in the metabolism cages, 1 cc. of the sample was injected. The twenty-four hour urine and washings from each cage were diluted to 1 liter and subdiluted to the proper concentration for the microbiological assay for calcium pantothenate. The Wood method (15) was used for calculating the results of the microbiological assays. The excretion values for the paired rats were 7.2, 7.4, 7.6, and 7.3 mg.; the average excretion per rat was therefore 3.69 mg. Correction for the "statistical" basal excretion of 0.13 mg. (v.s.) gave 3.56 mg. as the extra excretion due to the dose. When this figure was substituted for y in the formula for the dose-response curve, $y = -0.16 + 0.73x$; a potency of 5.1 mg. of panthenol per cc. was obtained. The standard error for this particular assay was calculated to be ±0.07 mg.

SUMMARY

An availability type of bioassay is presented for the determination of panthenol, the biologically active analog of pantothenic acid. The method is based upon the urinary excretion of pantothenic acid by the rat after dosage with panthenol. Pantothenic acid is determined microbiologically in the twenty-four hour urine. Specificity of the method has been demonstrated by comparison with rat curative and microbiological assays. Pantoic acid and pantoyl lactone do not interfere.

The dose-response relationship is essentially constant for rats varying in weight from 150 to 450 Gm. The usual working range (1–5 mg.) is sufficiently high to warrant the use of a statistically determined basal excretion. On a one-week dosage schedule, repeated administration of panthenol had no significant effect upon the level of basal excretion. The need for running concurrent standards or pre-dosage basal excretion has thus been eliminated.

The precision of the method can be judged by the fact that the average coefficient of variation $s/\bar{x}$, for 4-cage assays (8 rats) is 3 per cent.

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The Comparative Stability of Pantothenic Acid and Panthenol*

By SAUL H. RUBIN†

In vivo studies have shown that panthenol, the alcohol analog of pantothenic acid, displays the vitamin activity, qualitatively and quantitatively, of pantothenic acid. Under certain conditions of dosage, the physiological availability of panthenol is superior to that of pantothenic acid. The present *in vitro* experiments demonstrate that panthenol is considerably more stable than pantothenate in acid solutions at *p*H 3 to 5.

It is well known that the *p*H-stability pattern of pantothenic acid differs considerably from that of the other commonly used water-soluble vitamins which are sensitive to alterations in *p*H. Thiamine, riboflavin, and ascorbic acid are relatively stable in distinctly acid solution, become increasingly unstable as the reaction approaches neutrality, and are definitely unstable in alkaline solution. Pantothenic acid is most stable at *p*H *ca.* 6, increasingly unstable as the *p*H veers away to either side of 6 (1, 2). A detailed example of these opposing *p*H-stability relationships has been provided by Frost and McIntire (3), who determined the kinetics of hydrolysis of thiamine and pantothenate in acid solutions.

The practical difficulties inherent in this situation become sharply manifest in preparing *liquid* multivitamin preparations so as to provide maximum stability of the various components. The problem is more acute with regard to parenteral preparations because the permissible operating range of *p*H is somewhat circumscribed by the factor of pain on injection (4).

A plausible solution to this problem has been found in the pantothenic analog, panthenol, whose biological behavior is outlined in the preceding paper (5). Panthenol is more readily available than pantothenic acid when fed to the rat (5) and to man (6, 7) a circumstance which suggested greater stability of the former. This premise has been borne out by the comparative trials described in this report.

EXPERIMENTAL

Comparative stability trials were carried out both in pure solution—with added buffer and preservative—and in various multivitamin preparations.

Stability in Pure Solution.—Solutions of panthenol and sodium pantothenate at concentrations of 3 to 4 mg. per cc. were prepared in McIlvaine phosphate-citrate buffers (*ca.* 0.05 *M*) at graded *p*H from 3 to 8, and preserved with methyl *p*-hydroxybenzoate (0.02%) and propyl *p*-hydroxybenzoate (0.18%). The solutions were sealed in 5-cc. ampuls and kept in randomized distribution in a forced-draft incubator at 45 ± 0.5°. The use of phosphate-citrate buffer provided the *same* buffer anions at all *p*H, the only variable being the relative activities of buffer anions at different *p*H. This variable is not important in the present experiments, however, since the prime objective was to compare relative stability of the two compounds in pairs at equal *p*H.

The panthenol ampuls were assayed by the two bioassay methods described in the preceding paper (5), the curative and the excretion, and, in some cases, by a microbiological assay (8). Sodium pantothenate was determined by the curative bioassay, and by the microbiological procedure of Skeggs and Wright (9) with *Lactobacillus arabinosus*.

The results are summarized in Fig. 1. In order to segregate the results obtained by the curative bioassay, which was used for both panthenol and pantothenate, from the results by the other methods, the

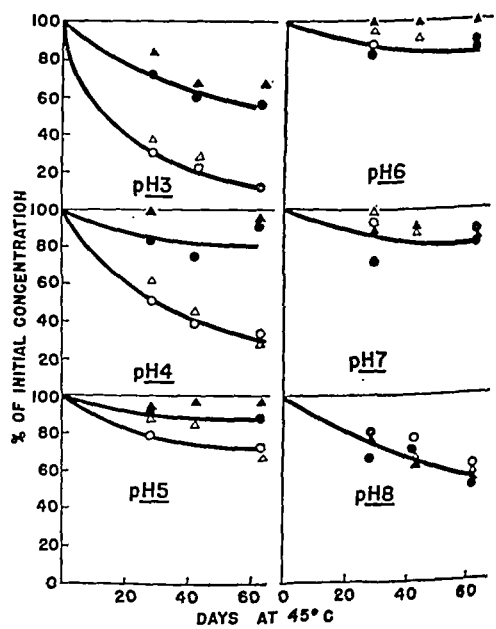

Fig. 1.—Comparative stability: panthenol and sodium pantothenate.

● Panthenol, curative bioassay in rats.,
▼ Panthenol, excretion bioassay in rats.
○ Na Pantothenate, curative bioassay in rats.
△ Na Pantothenate, microbioassay.

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† The author is indebted to Dr. M. F. Furter for advice and encouragement in this study, and to Dr. A. Motchane for discussions of certain aspects of the problem.

stability curves have been drawn through the former. Inspection of Fig. 1 shows clearly, however, that, irrespective of the assay method, there are considerable differences in the relative stability of panthenol and sodium pantothenate, panthenol being distinctly superior in the more acid solutions. The better stability of panthenol is most manifest at *p*H 3 and 4; at *p*H 5, the advantage is relatively slight, while at *p*H 6, 7, and 8, there are no apparent significant differences between the two.

The *p*H values shown in Fig. 1 are those of the solutions initially. After aging at 45° for 63 days, the panthenol ampuls showed little or no change in *p*H, while the more acid solutions of sodium pantothenate (*p*H 3, 4) became somewhat more alkaline, as detailed in Table I. This tendency to an alkaline shift is associated with the greater breakdown of pantothenic acid and the release of the amino group of β-alanine. It is apparent that this shift would favor the stability of pantothenic acid to the extent that the *p*H approaches 6, the *p*H of optimum stability. Obviously, however, it exerted no decisive effect on the relative panthenol-pantothenic picture in the present experiments.

TABLE I.—CHANGES IN *p*H OF PANTHENOL AND SODIUM PANTOTHENATE SOLUTIONS
*p*H determined with a Beckman glass electrode meter

Panthenol		Sodium Pantothenate	
Initial *p*H	*p*H after 63 Days at 45°	Initial *p*H	*p*H after 63 Days at 45°
3.0	3.1	3.0	3.5
4.0	4.0	4.0	4.3
5.1	5.2	5.1	5.2
6.2	6.2	6.2	6.2
7.0	7.0	7.0	7.0
7.8	7.6	7.8	7.6

	Assay	% Loss
Panthenol, pure solution	Excretion bioassay	3
Panthenol, hexavitamin solution	Excretion bioassay	5
Na pantothenate, pure solution	Microbiological	69
Na pantothenate, hexavitamin solution	Microbiological	76

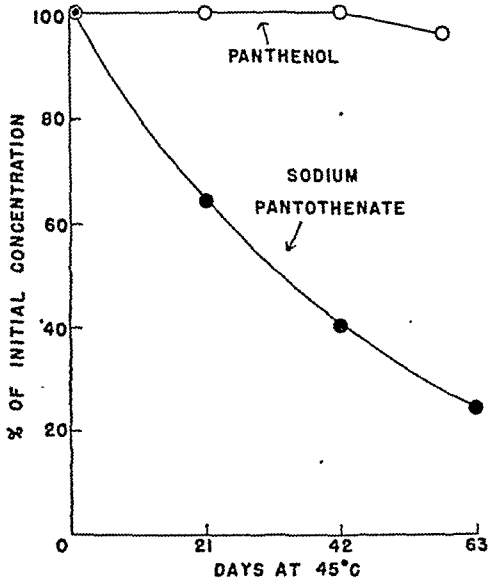


Fig. 2.—Relative stability of panthenol and sodium pantothenate in a liquid multivitamin preparation at *p*H 4.

Stability in Multivitamin Solution.—One of the more difficult stability problems presented by pantothenic acid occurs in liquid multivitamin preparations, which are usually made at *p*H *ca.* 4. Therefore, as a prime test of the comparative stability, an experimental hexavitamin preparation was studied containing thiamine, riboflavin, niacinamide, pyridoxine, ascorbic acid, and either panthenol or sodium pantothenate in equivalent amounts (*ca.* 5 mg./cc.). These were also aged at 45°. In these experiments, panthenol was determined by the excretion bioassay only, since, as has been demonstrated in the preceding paper (5) and in the experiments in pure solution detailed above, the two bioassay methods give essentially comparable results; the excretion bioassay is more precise, however. Sodium pantothenate was determined by microbiological assay with *Lactobacillus arabinosus*.

Figure 2 shows that the advantage of panthenol over pantothenate was maintained in this hexavitamin preparation, the loss of panthenol being nil up to forty-two days and possibly 5% at sixty-three days. The following tabulation of the values at sixty-three days at 45° delineates the close correspondence between relative stability in pure solution (Fig. 1) and in the hexavitamin preparation (Fig. 2); the presence of the other vitamins obviously did not alter the relative stability of panthenol or pantothenate.

Conversely, it was found that the other vitamins present—thiamine, riboflavin, niacinamide, pyridoxine, ascorbic acid—manifested the same stability irrespective of whether the preparation contained panthenol or sodium pantothenate.

The relative stability of panthenol and sodium pantothenate at *p*H 4 has also been studied at 60°. Figure 3 shows the results of a test of pure solutions at this temperature. Panthenol was determined by the excretion bioassay. Figure 3 represents essentially the same relative stability pattern as at 45°, and is further useful in showing that no critical temperature intervenes between 45° and 60°.

DISCUSSION

It is generally accepted that the first step in the breakdown of pantothenic acid is the splitting of the amide linkage between the lactone and β-alanine moieties. The work of Frost (2) on optical rotation as a measure of the stability of pantothenic acid supports this viewpoint. Frost and McIntire (3) showed that hydrolysis of pantothenate in acid solution follows a first-order reaction with respect to pantothenate concentration. Hydrolysis of pantothenate is therefore a less complicated process than, for example, the destruction of thiamine, which is subject to many influences other than *p*H, e.g., effect of buffer (10).

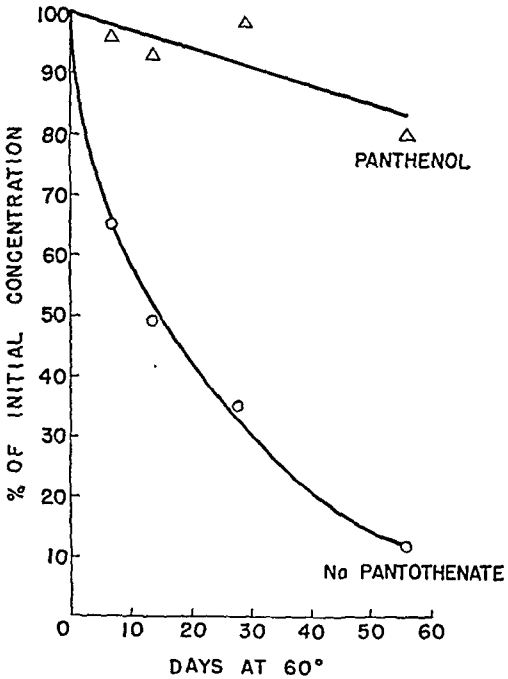
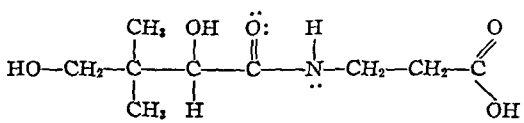


Fig. 3.—Comparative stability of Na pantothenate and panthenol at *p*H 4 and 60° C.

The greater stability of panthenol over pantothenate in acid solution appears to be associated with the lesser polarity of the terminal hydroxyl of panthenol as compared with the terminal carboxyl of pantothenic acid, and the relative influence of these on ease of hydrolysis of the amide linkage. The electronic configuration of the amide group in pantothenic acid may be formulated as follows:



It is well known that the first step in hydrolysis involves the addition of OH^- at the carbonyl carbon (transition state). This addition destroys the amide resonance. Conversely, weakening of the amide resonance makes the addition, i.e., hydrolysis, easier. Accordingly, the hydrolysis of amides is faster in acid solution than in alkaline (cf. 11).

The inductive effect of the terminal carboxyl of pantothenic acid, carrying a larger dipole than the terminal hydroxyl of panthenol, exerts a similar effect of increasing the positive charge on the nitrogen and thus weakening the resonance of the amide. The effect is particularly prominent in weak acid solutions where it is added to the effect of ammonium ion formation. (The amide being a very weak base, the ionization of N is not complete in the range of *p*H 3–4.) In alkaline solution (e.g., *p*H 8, Fig. 1), the resonance in the carboxyl group weakens the inductive effect on the nitrogen, and the rates of hydrolysis of pantothenic acid and panthenol become very nearly equal.

The better stability of panthenol in acid solution may also be involved in its superior physiological availability to rats and to humans (6, 7). The availability of pantothenic acid is diminished when taken with food (7, 12), whereas the availability of panthenol is not significantly affected (7). Silber (12) has shown in dogs that this effect on pantothenic acid is due, in part, to loss in the feces. However, a considerable portion of the pantothenic acid ingested is not accounted for. This unaccounted fraction may well be lost due to the greater acidity of the stomach after ingestion of food and consequent increased destruction of pantothenic acid.

The advantages of panthenol for multivitamin preparations are clearly apparent. Not only does panthenol provide greater stability in the commonly used range of acid *p*H, but, moreover, it becomes possible to drop the *p*H somewhat so as to provide a better measure of stability for thiamine, ascorbic acid, etc., with relatively little sacrifice of panthenol stability.

SUMMARY

The comparative stability of pantothenic acid and panthenol has been evaluated in buffered solutions over the *p*H range 3 to 8 and in multivitamin preparations. As determined by two independent methods for each vitamin, including a rat curative bioassay in each case, panthenol is considerably more stable at *p*H 3–4, somewhat more stable at *p*H 5, and about the same as pantothenate at *p*H 6–8. In a hexavitamin preparation at *p*H 4, the relative stability was the same as in pure solution at the same *p*H.

The implications of these data are discussed in relation to mechanism, vitamin formulation, and to the physiological availability of these vitamins.

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A Modification of the U. S. P. Method of Assay for Belladonna Extract*

By A. S. RIDOLFO† and E. P. GUTH‡

A new technique for the assay of extracted material is proposed which substitutes the use of sintered glass funnels for the Soxhlet apparatus in removing the alkaloids from the extract. This procedure is proposed as it was found that the thimbles used in the assay were adsorbing substances which reacted positively to Valser's Reagent.

A NEW technique for the assay of belladonna extract is proposed which substitutes the use of sintered glass funnels for the Soxhlet apparatus. Compared with the official method, this procedure features ease of operation, saving of time, and a high degree of precision and accuracy. Sixteen different samples of Extract of Belladonna were examined, both by the U. S. P. method and by a modified method, which indicated that the proposed method is advantageous.

It is recommended that this procedure be considered for the estimation of the alkaloidal content of extracts. It is recognized that the official assay for the alkaloidal content of solanaceous drugs presents difficulties. Fricke and Kaufman (1) investigated the effect of the quality of chloroform and the type of starch used in the extract as problems in the assay for Extract of Beladonna. Durret (2) suggested the use of neutralized alcohol in the final evaporation of the chloroformic solutions to retard alkaloidal decomposition during assaying. Others (3) have shown that keeping the alkaloids in an acid *p*H would increase racemization and hydrolysis.

Although precautions were taken in this work to follow the suggested remedies, erratic results were still encountered in assaying the Extract of Belladonna. Upon investigation of possible reasons for these discrepancies, the discovery was made that the Soxhlet thimbles were adsorbing substances which reacted positively to Valser's Reagent. Even after continuous percolation of fifteen hours according to the U. S. P. directions, acid washings of the thimble gave a milkywhite precipitate with Valser's Reagent. This prompted the possible use of the sintered glass funnel as a substitute for the Soxhlet apparatus.

DESCRIPTION OF APPARATUS

Equipment necessary for one unit:

1. A 250-ml. suction flask
2. A 50-ml. sintered glass funnel
3. A 50-ml. test tube
4. Porcelain crucible lid
5. A 5-ml. beaker
6. Suction tubing and screw clamps
7. Source of vacuum

The test tube is inserted into a 250-ml. suction flask, which is stoppered with a one-hole rubber stopper. The stem of the sintered glass funnel is extended through the stopper and into the test tube to a depth of about one inch below the side arm of the suction flask.

Any number of units may be attached to each other in a series by means of T-tubes. Clamps are so arranged that suction may be applied or removed from each unit. This is an important consideration for it permits the removal of the collected filtrates when necessary without disturbing the maceration or exhaustion of another unit and it permits the insertion of the 5-ml. beaker into the mouth of the test tube to receive the 1-ml. samples to test for the alkaloids (Fig. 1).

PROCEDURE

Approximately 3 Gm. of the accurately weighed extract is intimately mixed with two times its weight of washed sea sand (No. 20 mesh) and placed into the sintered glass funnel. Eighteen milliliters of the U. S. P. XIII alkaloidal extraction solution for solanaceous drugs is added and allowed to macerate for thirty minutes, keeping the funnel covered in order to prevent evaporation of the solvent. Slight suction is then applied by means of an aspirator and the contents of the funnel are sucked dry. The suction is then released and the collected filtrate is transferred from the test tube into a separatory funnel. This procedure is repeated four times using 10 ml. of ether and shortening the period of maceration to five minutes for each treatment.

Tests on 1-ml. samples of the filtrate with Valser's Reagent demonstrated the reliability of this method in exhausting the alkaloids.

The residue in the funnel is then washed with several 10-ml. portions of approximately *N*/5 H_2SO_4 until 1 ml. of the filtrate last collected is negative to Valser's Reagent. At this point the residue in the funnel should consist mostly of sand and starch with some extraneous matter. The acid washings are transferred to the separatory funnel containing the ether extracts and are used to extract the alkaloids from the ethereal solution. The remainder of the assay follows the official procedure

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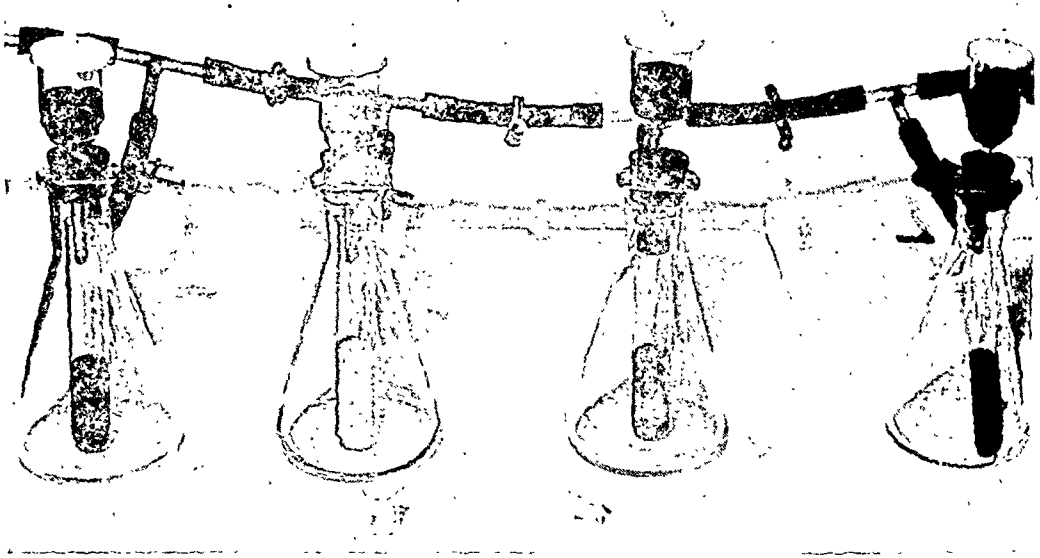

Figure 1.

except that it was found unnecessary to carry out the triple evaporation with chloroform as outlined in the official method. Instead a few milliliters of neutral alcohol was added at this point and the collected chloroform solutions were evaporated to dryness.

DISCUSSION

The results obtained and presented in Table I indicate the reliability of the substitution of a sintered glass funnel as a means of extracting the alkaloids of Belladonna from its extract. The use of a Soxhlet apparatus in the assaying of extracts is thought to be unnecessary since we are dealing with extracellular soluble material evaporated to dryness. By simple maceration and expression by suction, small quantities of solvent were found suitable to exhaust completely the alkaloids from the extract.

TABLE I.—ASSAY OF EXTRACT OF BELLADONNA BY U. S. P. AND MODIFIED METHOD[a]

Sample	U. S. P. Method	% Diff.[b]	Modified Assay	% Diff.
1	1.50	3.8	1.5	1.7
2	1.14	1.0	1.15	...
3	0.63	7.1	1.03	0.9
4	0.96	2.5	1.07	0.3
5	0.97	7.2	1.08	0.7
6	0.52	9.2	0.87	3.3
7	1.50	3.8	1.50	3.8
8	1.05	4.2	1.12	0.2
9	1.42	2.7	1.5	1.3
10	1.16	3.2	1.18	0.05
11	1.30	5.0	1.36	0.07
12	0.68	8.4	0.89	0.6
13	1.31	3.0	1.35	0.9
14	0.98	7.0	1.04	1.0
15	1.49	4.2	1.50	1.1
16	0.98	7.0	1.05	0.8

[a] Represents average of three assays for each sample.
[b] % difference is calculated on the highest and lowest values obtained for each sample.

The method proposed also eliminates the necessity for maceration overnight. This period of maceration is probably needed in assaying the crude drug, but since extracts of belladonna represent the active constituents removed from the drug in a concentrated form, overnight maceration is not necessary, especially since the alkaloids of belladonna are readily soluble in alcohol, ether, or chloroform.

From a study of the values presented, it may be seen that the use of a sintered glass funnel in the manner outlined produced higher results when compared to the U. S. P. method. This may be accounted for by the completeness of the extraction of the alkaloids, and also by the smaller quantities of solvent necessary for extraction.

SUMMARY

1. The substitution of a sintered glass funnel for the Soxhlet apparatus was found to be more efficient in the assay of Belladonna extract.

2. The time necessary to perform the assay is reduced to less than two hours.

3. Higher results are usually obtained.

4. Results are more uniform than those obtained by the U. S. P. method.

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Polarographic Determination of Folic Acid and Zinc Salts in Pharmaceuticals*

By JEANNE BUNDY DUNCAN and JOHN E. CHRISTIAN†

The quantitative estimation of zinc salts and folic acid by the polarographic method of analysis was investigated and the method was found to be applicable to pharmaceutical preparations. Barbituric acid was also studied, showing no oxidation or reduction at the dropping mercury cathode.

"POLAROGRAPHY" was developed as an analytical tool by Heyrovsky (1, 2) about twenty-five years ago. This electrochemical technique has been adapted in the ensuing years to almost every chemical and physical procedure. In certain cases, the simultaneous qualitative detection and quantitative determination of a number of different substances are possible from a single polarogram. The method is applicable over a range of 10^{-6} to 10^{-2} molarity, and best results are obtained at 10^{-4} to 10^{-3} molarity.

The basic concept of polarography lies in the principle that the electrolysis of an electrolyte in a cell activated by an easily polarizable dropping mercury electrode and a second large nonpolarizable electrode results in a current-voltage curve from which it is possible to determine both the nature and concentration of the reducible or oxidizable substance present.

Theoretically, any substance which is electroreducible or electrooxidizable within the potential range of the dropping mercury electrode can be analyzed by the polarographic method. In actual practice, this theory does not always hold true; nevertheless, the diverse applications of this procedure are of a wide variety.

A survey of the available literature reveals that the polarograph has not been used extensively in pharmaceutical analysis, but the possibilities in this field are almost unlimited.

EXPERIMENTAL

Apparatus and Methods.—This work was carried out with the use of the Sargent Model XX Heyrovsky Visible Recording Polarograph. The intersection point method of measuring wave height (diffusion current), as given by Müller (3) was used throughout the work. A capillary was used which had a $m^{2/3}t^{1/6}$ value of 1.684. Dissolved oxygen was removed from all solutions by bubbling hydrogen gas through the sample for at least five minutes. A mercury pool on the bottom of the electrolysis vessel served as the large nonpolarizable electrode.

Zinc Salts.—There are many references made to the determination of the zinc ion in the literature; however, there is no record of the application of the polarographic method to pharmaceutically important zinc salts.

Standard quantitative curves for several zinc salts were determined. Zinc chloride and zinc acetate of the N. F. VIII, in addition to zinc sulfate, zinc oxide, and zinc stearate, which are official in the U. S. P. XIII, were used. A solution of 0.1 molar ammonium acetate at its original *p*H of 6.75 was used as the supporting electrolyte. No maximum suppressor was required.

Samples were prepared for producing standard curves of the zinc salts by diluting the required volume of a 0.05 *N* solution of the salt with 0.1 molar ammonium acetate solution.

Since zinc chloride is very hygroscopic, it was dried at 110° for twenty-four hours before weighing. This procedure converts the $ZnCl_2$ to Zn_2OCl_2. Zinc oxide was treated with the minimum amount of diluted HCl necessary to cause solution before preparing the sample by the general procedure. Zinc stearate was placed in a crucible, and the fatty acids were burned off in an electric muffle furnace. The residual zinc oxide was then dissolved as described above.

These solutions were electrolyzed at a temperature of 25° with the sensitivity setting on the polarograph of 2–98 and a damping control of 3. The Initial Potential was 0 volt, and the Span Potential was 2 volts.

Table I shows the diffusion currents obtained for these solutions.

TABLE I.—AVERAGE[a] I_d OF ZN SALTS[b]

Concn.	Zn_2OCl_2	$ZnSO_4$	Zn Acetate	ZnO	Zn Stearate
0.00100 *N*	16.75[c]	18.86	18.10	17.44	15.90
0.00075 *N*	12.37	14.28	13.64	12.85	11.92
0.00050 *N*	8.52	9.79	9.43	8.79	8.96
0.00025 *N*	4.38	4.93	5.00	4.54	4.57
0.00010 *N*	1.90	2.06	2.01	1.81	1.75

[a] Average of from six to twelve determinations.
[b] In 0.1 *M* ammonium acetate.
[c] I_d expressed in microamp.

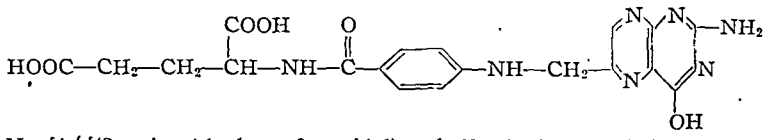
The average wave heights for each concentration shown in Table I when plotted on a graph as ordinate against concentration as abscissa produce a straight line, which can be used as a standard curve for determining unknowns of the same zinc salt.

Barbituric Acid.—The polarographic analysis of barbituric acid would seem dubious, since the literature indicates that the parent compounds do not react at the dropping mercury electrode. This ex-

* Received April 12, 1948, from the School of Pharmacy, Purdue University, Lafayette, Ind.
† The authors express their appreciation to the Lederle Laboratories division of the American Cyanamid Company for supplying the samples of folic acid.

perimentation revealed that barbituric acid gave no polarographic wave in supporting electrolytes of 0.1 molar ammonium chloride, 0.2 molar tetraethylammonium bromide, or in buffers ranging from *p*H 3 to 10.

Folic Acid.—One of the newer members of the vitamin B Complex group, folic acid, has recently been synthesized. The accepted structure for the Liver *L. casei* Factor or Folic Acid is (4):



N—[4-{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}benzoyl]-glutamic acid
Pteroylglutamic Acid

The above molecule shows several groupings which might lend themselves to polarographic analysis.

At the present time, a microbiological assay is used for the determination of folic acid (5).

A sample of 87.7% pure folic acid was obtained for experimentation. An unbuffered solution made by dissolving folic acid in 10% ammonium hydroxide with a supporting electrolyte of 0.1 molar ammonium chloride was electrolyzed with no results.

Buffer solutions were next prepared according to Kirkpatrick (6) and the U. S. P. (7). The concentration of the salts in the buffer solutions was great enough so that the buffer would also act as the supporting electrolyte.

Samples were prepared by treating the folic acid with the minimum amount of 10% ammonium hydroxide necessary to cause solution, and diluting with water to make a standard solution of 0.005 molarity. To the correct volume of folic acid standard solution was added 50 ml. of buffer, and the entire solution was then made up to 100 ml. with distilled water.

It was found that folic acid was not soluble in glacial acetic acid in the concentration necessary for polarographic analysis. Therefore, the alkaline buffers are much more efficient in the already alkaline solution of folic acid.

A preliminary investigation revealed that folic acid in buffered solutions of *p*H range of 3–10 gives a polarographic step. It was discovered that solutions having a *p*H of 6.5 or lower produce two polarographic waves; the first wave is usually proportional to concentration. The *p*H range of 8–10 was found to give the best results. Therefore, a series of folic acid solutions buffered to a *p*H of 9 with a buffer containing boric acid, potassium chloride, and sodium hydroxide (7) was selected to determine whether wave height (diffusion current) was directly proportional to the concentration of folic acid present in the solution. The diffusion currents expressed in microamperes resulting from these solutions are listed below.

Concentration	Microamperes
0.0001 *M*	2.18
0.00025 *M*	5.55
0.0005 *M*	10.90
0.00075 *M*	16.24
0.001 *M*	21.60

Each figure is an average of approximately seven different determinations. These solutions were electrolyzed at a temperature of 25° with a sensitivity setting on the polarograph of 2–95 and a damping control of 3. The initial potential was 0 volt, and the span potential was 3 volts.

These diffusion currents when plotted on a graph as ordinate against concentration as abscissa give a straight line slope. We can assume that under these experimental conditions the diffusion current of folic acid is a linear function of concentration.

The fact must be kept in mind that this standa curve was prepared using folic acid of 87.7% purit

A typical polarogram of folic acid is shown Fig. 1. This is a current-voltage curve of 0.000 molar folic acid in a solution buffered to *p*H 9.

The half-wave value of the total applied E. M. for folic acid in a buffered solution of *p*H 9 appea to be approximately −0.87 volt. This half-wa potential seems to be independent of concentratio

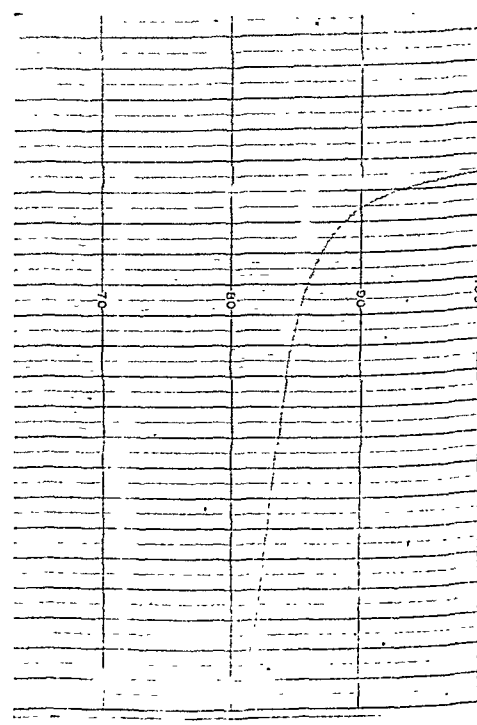


Fig. 1.—Polarographic wave of 0.00075 *M* folic acid

The half-wave value does become slightly mor negative with increasing *p*H, but the increase is no great enough to show that hydrogen ions are use or given off in the electrode reaction.

Solutions of folic acid buffered to a *p*H of 1 gave a wave with a maximum-like characteristi

which resulted in an irregular limiting current quite difficult to measure with accuracy. This maxima was eliminated in solutions containing only 0.002% gelatin, but the resultant wave heights are not proportional to concentration. Methyl red had no suppressive effect on this maxima. Addition of ions such as potassium, barium, and calcium were not helpful in obtaining a more regular wave. Where ions with higher valences were added, the half-wave potential seemed to be slightly more negative.

The procedure was also applied to a limited number of commercial tablets containing folic acid with good results.

SUMMARY

Various substances of pharmaceutical importance were investigated for the applicability of their determination by means of the polarographic method. Quantitative analysis was stressed in all cases.

Zinc chloride, zinc sulfate, zinc acetate, zinc oxide, and zinc stearate can be quantitatively determined by polarographic analysis in a supporting electrolyte of 0.1 molar ammonium acetate in concentrations ranging from 10^{-3} to 10^{-4} molarity.

The possibility of reducing the time required for barbiturate analysis, if the polarographic method could be applied to barbituric acid and its derivatives, was investigated. The experimentation revealed that barbituric acid gave no polarographic wave in supporting electrolytes of 0.1 molar ammonium chloride, 0.2 molar tetraethylammonium bromide, or in buffers ranging from *p*H 3 to 10.

The conditions under which folic acid can be quantitatively determined by the polarographic method have been described in detail. Best results were obtained when the aqueous solution of folic acid was buffered to a *p*H of 9 with a buffer solution containing boric acid, potassium chloride, and sodium hydroxide. The resultant half-wave value of the total applied E. M. F. is approximately −0.87 volt. Under these experimental conditions, the polarographic method was shown to give reproducible results for folic acid present in concentrations ranging from 10^{-3} to 10^{-4} molarity. This assay was also applied to a limited number of tablets containing folic acid with good results. The possibility of the application of this quantitative polarographic procedure to folic acid preparations is apparent.

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Urine Iodine Following Ingestion of Different Iodine Preparations*.

By J. C. FORBES

It is generally agreed that the urinary excretion of iodine following the oral administration of a single dose of iodized fatty acids is considerably slower than after the ingestion of sodium or potassium iodide. Bröking (1) found on the average 75 per cent of the iodine of a single dose of potassium iodide in the urine during the next twelve hours. On the other hand, only 35–50 per cent of the iodine of Sajodin (calcium iodobehenate) was excreted in the urine by the eighty-fourth hour. Seven to 10 per cent of the latter preparation was excreted in the feces while only traces of the former were excreted by way of the bowel. Bonanni (2) found urinary iodine excretion to reach a maximum in about three hours following the ingestion of sodium or potassium iodide while it required seven to eight hours for maximum urinary excretion to be reached following the administration of iodized fatty acids. Bröking (1) showed that maximum excretion following the ingestion of iodized fatty acids might not be reached until the twelfth hour. Osborne (3) showed that the rate of iodine excretion in the urine was independent of urine volume, and reached a maximum in two to four hours following the administration of potassium iodide. McLean (4) found more iodine in the fatty tissues

* Received June 16, 1948, from the Department of Biochemistry, Medical College of Virginia, Richmond, Va.

of the body of rabbits following the administration of calcium iodobenhenate than after an equivalent amount of potassium iodide. Peabody (5) found no consistent difference in the iodine content of various body tissues of rabbits following the oral administration of potassium iodide and of ethyl diiodobrassidate over a considerable period of time. However, total iodine retention was a little greater in the case of the iodized fatty acid derivative.

Since there is no marked storage of iodized fatty acids in the body and since they are well absorbed from the gastrointestinal tract, repeated dosage with these iodized fatty acids should lead finally to approximately the same urinary iodide excretion per twenty-four hours as after an equivalent amount of sodium or potassium iodide. The present investigation was started to determine if this was the case and if the rate of excretion would be more constant in the case of the iodized fatty acids than in the case of the inorganic iodides.

EXPERIMENTAL

Five iodine preparations have been studied: potassium iodide, ethyl diiodobrassidate, calcium monoiodostearate, a calcium salt of mixed iodized fatty acids obtained by the fractional distillation of the fatty acids of hydrolyzed linseed oil, and stearolic acid diiodide. The preparation obtained from linseed oil undoubtedly was a mixture of mono-, di-, and triiodo fatty acids with possibly some saturated acids also. Its iodine content was only 33%. No free or inorganic iodine could be demonstrated in any of the organic preparations studied.

Urinary iodine was determined by the method of Gerard and Raunet (6). Preliminary experiments with the calcium salts of the iodized fatty acid showed that, for maximum absorption, they should be administered in a finely divided state. Consequently, in all the experiments reported, the organic iodine preparations were ground with corn starch and a weighed amount of the finely divided mixture put into hard gelatin capsules for oral administration. Although a number of subjects were used in studying some of the preparations, the results presented were all obtained on the same subject, the work extending over a period of a year. The results obtained with the stearolic acid diiodide are not included as they were very similar to those obtained with ethyl diiodobrassidate.

Figure 1 shows the urinary iodine excretion following the oral administration of a single dose of the various preparations. It will be seen that the iodine excretion reached a maximum earlier after potassium iodide than after any of the other preparations. The calcium salt of the mixed iodized fatty acids was next. In one subject, not shown in the charts, urinary iodide excretion following the ingestion of ethyl diiodobrassidate closely approximated that following the ingestion of the mixed iodized fatty acids, but this was usually not the case. The percentage of the total iodine intake excreted in the urine during the first twenty-four hours following the

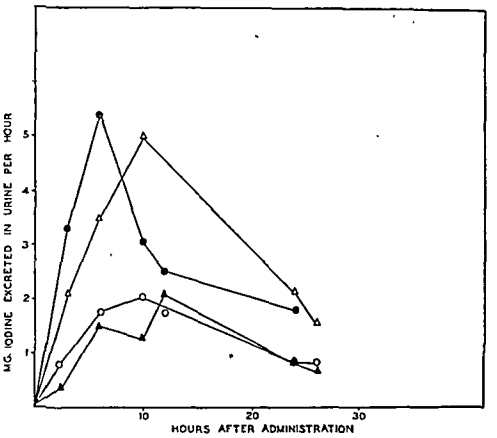

Figure 1.

⊙—After 88 mg. of iodine as KI. Average 2 exp.
△—After 86 mg. of iodine as Ca salt of mixed iodized acids. Average 2 exp.
○—After 86 mg. of iodine as Ca monoiodostearate. Average 2 exp.
▲—After 77 mg. of iodine as ethyl diiodobrassidate. Average 4 exp.

ingestion of a single dose of calcium monoiodostearate, ethyl diiodobrassidate, and stearolic acid diiodide was much less than in the case of potassium iodide or of the calcium salt of the mixed iodized fatty acids. However, when the various preparations were administered for several days the difference in total urinary iodine excretion per twenty-four hours became quite negligible (Figs. 2 and 3). It will be noted that after iodine administration for

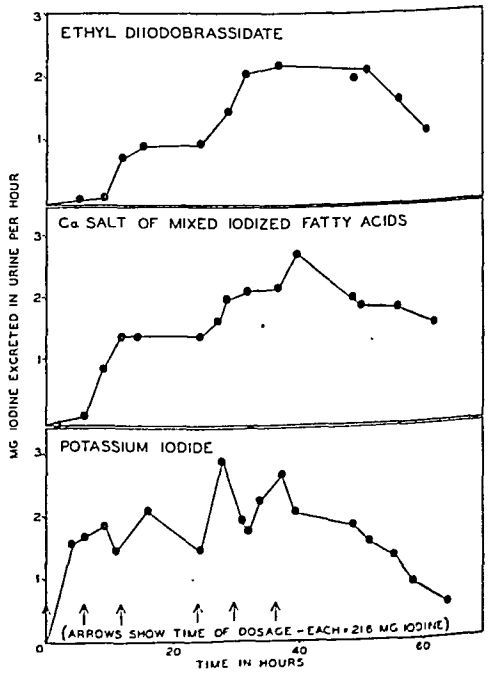

Figure 2.

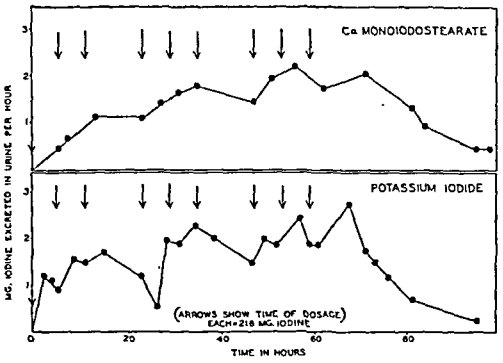

Figure 3.

a couple of days the urinary excretion rate became considerably more constant in the case of the organic iodine preparations than in the case of potassium iodide. As far as could be determined, all of the iodine in the urine was present as the iodide. Repeated attempts to show the presence of iodized fatty acids in the urine failed, even during the periods when urinary iodine elimination was at a maximum.

Since the rate of iodine excretion in the urine, in the case of a normal functioning kidney, must reflect the level of inorganic iodine in the plasma, it is safe to assume that the inorganic iodine level of the plasma obtained from the administration of repeated doses of iodized fatty acids would be more constant than from the administration of inorganic iodides. Such a result would also be expected from the fact that inorganic iodides are rapidly absorbed and eliminated, while, in the case of the iodized fatty acids, the liberation of inorganic iodine into the plasma is a function of the absorption and rate of metabolism of the iodized fatty acid. In view of this, it would appear that iodized fatty acids should offer definite therapeutic advantages over inorganic iodides in the treatment of such conditions as arteriosclerosis and hyperthyroidism. A recent article by Wolff and Chaikoff (7) is of considerable interest in this connection. These authors have shown that as long as the plasma inorganic iodine level is kept above 20–35 gamma per cent, organic binding of iodine by the thyroid gland is blocked, but as soon as the level drops below this critical level, formation of thyroxine and diiodotyrosine begins again. The more constant maintenance of this critical level without danger of excessive concentrations being reached at times should be considerably easier with the administration of iodized fatty acids than with inorganic iodides.

SUMMARY

The excretion of iodine in the urine following the oral administration of potassium iodide, ethyl diiodobrassidate, calcium monoiodostearate, stearolic acid diiodide, and the calcium salt of mixed iodized fatty acids prepared from the fatty acids of linseed oil has been studied.

Following the oral administration of a single dose, urinary iodine excretion was more rapid after potassium iodide than after the iodized fatty acids. However, when repeated doses of the various preparations were administered over several days, urinary excretion per twenty-four hours became roughly the same for all of the preparations studied.

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Artificial Inoculation of Rye with Ergot. A Susceptible Rye and the Control of Ergot Beetles*

By RALPH W. LEWIS

A strain of tetraploid Rosen rye was shown by artificial inoculation to be very susceptible to ergot. The ergot beetle, whose larvae eat the developing sclerotia, can be largely controlled by the use of DDT dust.

IN A previous paper (1) and in a patent (2) the author has described a method for the artificial inoculation of rye with ergot. The novelty of the method consists of spraying the blooming rye plants with a heavy sugar-spore-suspension rather than a plain water suspension of spores. The spores in droplets of a sugar suspension remain viable and capable of causing infection for several days after being sprayed on the rye spikes. Spores in a plain water suspension lose their ability to germinate in a short time and thus

* Received May 18, 1948, from the Department of Biological Science, Michigan State College, East Lansing, Mich.

can only cause infection if they fall on a stigma when first sprayed on the rye.

The possibility of making this method commercially feasible depends upon the determination of the optima of several variables as noted previously (1). One of the variables is the variety of rye employed. This paper describes a strain of rye which, because of its susceptibility and habit of growth, appears to be more desirable than Common Rosen rye (*Secale cereale*) for the production of ergot. Results on the use of DDT to control the ergot beetle, whose larvae do considerable damage to the developing sclerotia, are also reported. Due to various circumstances this research was terminated before all the planned experiments were carried out, consequently the results are fragmentary and all conclusions tentative.

EXPERIMENTAL

In 1944 a number of varieties of rye were tested for a susceptibility to ergot by spraying them with a sugar-spore-suspension. One variety called Double Rosen, a tetraploid, was much more susceptible than the others. It had 48% of the heads infected with at least one sclerotium while the sprayed Common Rosen, a diploid, had 28% and the unsprayed Common Rosen had less than 1%. These trials consisted of 15-ft. rows of each variety repeated four times. They were sprayed only once.

The Double Rosen rye was secured from Dr. H. H. Love, Department of Agronomy, Cornell University. It was different from Common Rosen rye in that many of the florets did not produce seed. Because the spike was light due to the failure of many seeds to ripen and because the straw was stiff, the spike remained upright even after the seeds and sclerotia had matured. The uprightness of the spike would be a desirable trait if a mechanical harvester were to be used to gather the sclerotia direct from the field. The partial sterility of this strain of Double Rosen rye makes available a larger amount of food for the developing sclerotia and it appears to cause the flowers to remain open longer, thus exposing them to infection for a longer period of time. The stigmas of the Double Rosen were longer than those of the Common Rosen rye. The average length of stigma (five of each measured) was 5.8 mm. for the former and 4.3 mm. for the latter. Considering that the spores germinate on the stigma to bring about infection this extra length is important. More of the stigma is exposed at the time of blooming and much more remains exposed to the outside by protruding between the palea and lemma after the flower closes.

A second lot of the Double Rosen seed was requested from Dr. Love for the 1945 season. A large planting was made from this new seed. The seed proved to be different from that used the previous year. The plants appeared identical with Common Rosen, that is, the spikes all drooped when ripe and the stems were not so robust. All comparative results between Common and Double Rosen for this season were destroyed by burning of the plants with arsenate dust or by wind. Observations in the field, however, led to the conclusion that there was little difference in the amount of infection in this lot of Double Rosen as compared to the Common Rosen rye. The previous year's results were so pronounced that the difference was obvious in the field before the plants were harvested, thus large differences in yield of sclerotia can be recognized by field observation.

In order to use the strain of rye that gave such good results in 1944, seed from the original lot was planted for inoculation in 1946. Small plots 6 rows wide and 15 ft. long were laid out adjacent to each other along the same 6 rows. Plot 1 was sprayed three times in the same morning with a high quality (50% germination) spore suspension diluted 1–15 as described in a previous paper (1). Plot 2 was unsprayed and Plot 3 was sprayed twice with the same spore suspension in the late afternoon five days after Plot 1. Both plots were sprayed during the seven- to nine-day blooming period in early June. As soon as the "honey-dew" began to be evident, which was about fifteen days after inoculation, ergot beetles (*Acylomus ergoti* Casey) were present by the hundreds. The north three rows of each plot were dusted once with 5% DDT.

RESULTS AND DISCUSSION

Results of three plots selected as typical are presented in Table I. It will be noted that the control plot has a high percentage of ergot infection as compared to the control plots reported previously (1). This can be expected to occur when dealing with small plots of a very susceptible rye. The longer blooming period of this rye, the larger number of insects that transmit the spores, and the blowing of the spore suspension probably account for this large percentage of infection in the control plots. There is, however, 20 to 40 per cent more infection on the treated plots. The highest infection of Common Rosen rye obtained in previous work (1) was 60 per cent in the treated plots with 10 per cent in the untreated plots. This high percentage of infection on the Common Rosen rye was secured by spraying the plants on five different days with a spore suspension approximately 15 times as concen-

TABLE I.—THE EFFECT OF DDT UPON THE ARTIFICIAL INOCULATION OF DOUBLE ROSEN RYE WITH ERGOT

Plot	No. Times Sprayed	Insecticide	Per Cent Spikes Infected with Ergot
1*a*	3	DDT	88
1*b*	3	None	89
2*a*	Control	DDT	50
2*b*	Control	None	47
3*a*	2	DDT	73
3*b*	2	None	72

trated as used in the work described in the present paper. The Double Rosen was more highly infected despite fewer applications of spores in a much greater dilution.

The DDT had no effect on the number of sclerotia which developed in each plot, but it did have a pronounced effect upon the number of insects as measured by their effect on the sclerotia. The samples from 1*a*, 2*a*, and 3*a* were thrown together as were 1*b*, 2*b*, and 3*b*. The percentage of sclerotia with insect burrows was 68 for the undusted plots and 14 for the dusted plots. This difference is paralleled by a field count of 28 adult beetles on two undusted rows while there were only 2 beetles on comparable dusted rows.

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Influence of *p*H on the Diastatic Activity of Fungal and Malt Amylases*,†

By R. S. J. RAU and A. SREENIVASAN

The effects of changes in *p*H on the diastatic activity of fungal and malt amylase preparations are reported and the bearing of the results on their use as pharmaceuticals is discussed.

A NUMBER of workers (1) have investigated the action of amylases at various hydrogen-ion concentrations for studying the optimum *p*H ranges over which they are active and, while it is generally recognized that amylases are inactive at high alkaline and particularly acidic *p*H ranges, no work has been reported on the effects of a sequence of *p*H changes similar to that existing in the gastric and duodenal regions. Such a study is of significance especially with fungal and malt amylases which have been the commonest among the diastatic preparations used in medicine (2). Takamine (3) has referred to the relatively greater stability and resistance to acid of the amyloclastic enzyme of taka-diastase as compared to that of malt. Ohlsson and Swaetichin (4) have observed loss in power of taka-diastase to hydrolyze starch in acid ($<$ *p*H 2.0) or alkaline ($>$ *p*H 11.5) solutions and suggest that reactivation or regeneration of the enzyme may take place on restoration of the *p*H to the optimal value by neutralization. The mechanism of inactivation of taka-diastase has also been studied by Yamashiki (5) and, more recently, by Akabori, *et al.* (6); the latter authors report that the amylase is inactivated in thirty minutes at *p*H 2.0, that it is reactivated by adjustment to *p*H 5.0 and that, therefore, activation and reactivation are primarily reversible processes.

In order to investigate this property further under conditions more or less similar to those *in vivo* and also to provide data which might serve as a basis for the therapeutic use of fungal and malt amylase preparations, the following studies were undertaken.

EXPERIMENTAL

Materials and Methods.—The amylase preparations employed in these studies were both prepared in the laboratory: for the *fungal amylase*, a strain of *Aspergillus oryzae*, No. 558 from the National Collection of Type Cultures, Bangalore, India, was inoculated on a previously cooked and cooled bran medium and growth allowed to proceed in shallow trays at 30° for two and a half days. The moldy bran was then extracted with ten volumes of 2.5% saline and the enzyme precipitated from the filtered extract by addition of alcohol to a concentration of 70%. *Malt diastase* was obtained by extraction with water of three-day germinated barley at room temperature (28°) followed by precipitation of the enzyme from solution with 70% alcohol. The fungal and malt enzyme preparations obtained as above were purified by dissolution in water followed by reprecipitation from 70% alcohol; the dry products had activities, respectively, of 16,300 and 13,500 Lintner units per gram (7).

Saccharogenic activities of the enzyme preparations, under specified conditions, were measured by the iodometric determination of reducing sugars (8) formed after half-hour hydrolysis at 37.5° acting on 2% maize starch solution. Suitable controls were always run and the reducing values recorded are those obtained after allowance for blank readings.

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† Acknowledgment is made to the Provincial Industrial Research Committee of the Bombay Government Department of Industries for a grant in support of this work.

Influence of *p*H on Amylase Activity.—The saccharifying power of the enzyme preparations was studied at *p*H 1.8, corresponding to the concentration of gastric hydrochloric acid, at *p*H 3.4 at which *p*H *alpha*-amylase is known to be inactivated (9, 10), at *p*H 4.8, the optimum *p*H for both the enzymes, and, finally, at *p*H 8.0, corresponding to the *p*H of the intestinal secretions. The buffer solutions employed were Clark's *M*/5 potassium chloride-hydrochloric acid (1.8), Clark's *M*/5 potassium hydrogen phthalate-hydrochloric acid (3.4), Walpole's *N*/1 sodium acetate-acetic acid (4.8), and Sorensen's *M*/15 phosphate (8.0). The reaction mixture consisted of 10 ml. of 4.0% starch solution, 2 ml. of the appropriate buffer solution, 3 ml. of water, and 5 ml. of enzyme solution containing 2 mg./ml. of the dry preparation. The results (Table I) are expressed in terms of milligrams of maltose hydrate per 100 ml. of the starch-enzyme mixture.

TABLE I.—ACTIVITIES OF FUNGAL AND MALT AMYLASES AT VARIOUS *p*H

	Mg. Maltose Hydrate Formed per 100 Ml. of Reaction Mixture After 1/2 Hr. Digestion			
	*p*H 1.8	*p*H 3.4	*p*H 4.8	*p*H 8.0
Fungal amylase	68	136	408	102
Malt amylase	0	85	323	119

It may be seen that there is pronounced loss in enzyme activity on either side of the optimum *p*H; the decrease in activity is more in acid ranges and, with malt amylase, there is complete inactivation at *p*H 1.8.

Rate of Inactivation at *p*H 1.8.—The decrease in activity in presence of acid (Table I) may be due to either adverse effect of *p*H or to inactivation of the amylases. To ascertain this further, a study was made of the rate of loss in enzymic activity at *p*H 1.8. Eighteen milliliters of the enzyme solution, containing 5 mg. of dry preparation per ml., were diluted with 24 ml. of water and treated with 3 ml. of buffer (*p*H 1.8). At stated intervals, 5-ml. aliquots of the reaction mixture, corresponding to 10 mg. of the enzyme preparation, were pipetted into a mixture of 10 ml. of 4% starch solution and 5 ml. of buffer (*p*H 4.8). The saccharogenic activity was measured as before. The results for fungal amylase are given in Table II.

It is interesting to note that, although fungal amylase loses a considerable percentage of its activity at *p*H 1.8 for half an hour (Table I), the activity returns to almost the normal value on restoration of the *p*H to the optimum for the enzyme. There is, however, slow and stepwise degradation of the enzyme on continued contact at low *p*H but there is little doubt that inactivation at acid *p*H ranges is largely of a reversible type.

When these experiments were repeated with malt enzyme, the striking observations were made that malt amylase was almost instantaneously inactivated at *p*H 1.8 and that this inactivation was irreversible, there being no activity even after restoration to optimum *p*H. It was therefore obvious that malt amylase was irreversibly destroyed at highly acidic *p*H.

Action of Pepsin.—Enzymes being in the nature of proteins, the activities *in vivo* of the amylase preparations may, it was felt, be adversely affected by the pepsin of the stomach. In the following experiments, peptic digestion of 5-ml. lots of mold and malt amylase solutions containing 5 mg. of enzyme per ml. was carried out at *p*H 1.8 and at 37.5° by addition of 1 ml. of buffer (1.8) and 5 ml. of a solution of pepsin, containing 6 mg./ml. of a commercial preparation (B. D. H.) and after dilution to 15 ml. At the end of half an hour, saccharogenic activity was determined as usual by pipetting out 5 ml. of the reaction mixture, corresponding to 10 mg. of the amylase, into a mixture of 10 ml. of 4% starch solution and 5 ml. of buffer (*p*H 4.8). Dialysis of the peptic digests was tried before adjustment to *p*H 4.8 but the alternative procedure detailed here was found to be easier and more satisfactory, the desired *p*H change being effected by suitable adjustment of the quantities of the buffers as indicated above. The results together with those

TABLE II.—RATE OF INACTIVATION OF FUNGAL AMYLASE AT *p*H 1.8

Time of contact of the enzyme with the buffer (*p*H 1.8) in minutes	0	2 1/2	5	10	20	30
Milligrams maltose hydrate formed per 100 ml. of reaction mixture at end of 1/2 hr., *p*H 4.8, 37.5°	391	374	374	357	357	340

of control experiments at *p*H 1.8 without pepsin and at optimum *p*H are presented in Table III.

TABLE III.—ACTION OF PEPSIN ON AMYLASES

	Mg. of Maltose Hydrate Formed per 100 Ml. of Reaction Mixture After 1/2 Hr. Digestion	
	Fungal Amylase	Malt Amylase
Amylase + pepsin (*p*H 1.8 for 1/2 hour followed by *p*H 4.8)	357	51
Amylase only (*p*H 1.8 for 1/2 hour followed by *p*H 4.8)	357	0
Amylase only at *p*H 4.8	357	316

The results show that pepsin does not affect amylase activity. In fact, malt amylase is slightly protected by pepsin against inactivation by acid, retaining thereby about one-sixth of its original activity.

Action of Pancreatin.—As fungal amylase was regenerated after inactivation by acid, the activity of the amylase was studied under conditions similar to those prevailing in the small intestine. The combined action of fungal and pancreatic amylases was also studied at *p*H 8.0 (Table IV). The reaction mixture consisted of 10 ml. of 4% starch solution, 5 ml. of buffer (*p*H 8.0), and 5 ml. of enzyme solution containing 2 mg./ml. of fungal amylase or malt amylase with and without pancreatin (Stearn's) in similar amount.

TABLE IV.—ACTION OF PANCREATIN

	Mg. of Maltose Hydrate Formed per 100 Ml. of the Reaction Mixture After 1/2 Hr. Digestion
Fungal amylase	102
Pancreatin	34
Fungal amylase + pancreatin	187
Malt amylase	119
Malt amylase + pancreatin	204

As in the case of pepsin, there is evidently a protective effect exerted by pancreatin on the diastassis of starch by fungal or malt amylase at *p*H 8.0. The possible mutual synergism between these amylases also should not be overlooked.

That there could be pronounced synergism between amylases from different sources was shown in other studies, to be published elsewhere, using highly purified laboratory preparations of amylases of vegetable, animal, bacterial, and fungal origin, either singly or in combination. A summarized statement of the results obtained in a typical set of experiments is given in Table V. The reaction mixture consisted of 50 ml. of 4% starch solution, 10 ml. of appropriate buffer, and 10 ml. of enzyme solution containing 10 mg. of the enzyme product or mixture of enzyme products per ml., finally made up to 100 ml. with water. Hydrolysis was carried out either at the optimum *p*H in the case of individual enzymes or at a suitable intermediate *p*H for mixtures; this had to be done since pancreatic amylase had very low activity at *p*H 4.8. Maximum saccharification was reached in seventy-two to one hundred and forty-four hours in all cases. The limit of saccharification recorded in Table V represents the maximum of the reducing sugars formed expressed as per cent on the value obtained by hydrolysis of starch solution with concentrated hydrochloric acid for one hour at 15 lbs. steam pressure (125°); this latter treatment is known to result in complete breakdown of starch and dextrins to reducing sugars (11).

TABLE V.—SACCHARIFICATION OF STARCH BY AMYLASES

Enzyme or Enzymes	*p*H of Hydrolysis	Period of Hydrolysis, Hr.	Limit of Maltose Formation, %
Fungal amylase	4.8	96	57.8
Malt amylase	4.8	72	69.3
Pancreatic amylase	7.0	144	47.0
Fungal amylase + malt amylase (9:1)	4.8	72	65.3
Fungal amylase + pancreatic amylase (1:9)	5.8	120	67.9
Malt amylase + pancreatic amylase (1:9)	5.8	72	68.8

DISCUSSION

Inactivation of malt amylase by acid, especially in concentration similar to that of gastric hydrochloric acid immediately after food ingestion, is almost instantaneous and is, besides, irreversible. It would therefore follow that, except in so far as they provide predigested carbohydrate and protein foods, preparations of malt have little value as diastatic agents in medicine and that there is no practical significance in the determination of diastatic value of malt extracts as laid down in official methods (12). The value of malt diastase, when employed with enteric coatings that disintegrate in the duodenum (13), is also perhaps limited because of low activity in the *p*H range of the duodenum.

With fungal amylase, on the other hand, though there is a certain amount of slow and stepwise inactivation at low acidic *p*H, the enzyme is quickly regenerated on restoration of *p*H to its optimum. Even at *p*H 8.0, there is improved activity due to the synergistic effect of pancreatic amylase.

The change in reaction from acidic to alkaline side, during the period the food material passes from the gastric to the duodenal region, may be expected to be gradual, passing through the optimal range for the fungal amylase; hence, it may be expected that the cumulative action of the enzyme will be considerably more than *in vitro* digestibility trials, such as those reported here, would indicate.

SUMMARY

1. Diastatic activities of fungal and malt amylases are affected adversely on either side of the optimum *p*H 4.8. Malt amylase loses its diastatic power at *p*H 1.8.

2. Malt amylase is irreversibly inactivated almost instantaneously at *p*H 1.8, whereas with fungal amylase at low acid *p*H ranges inactivation is slow and stepwise besides being reversible. Thus, the enzyme could be regenerated by restoration of *p*H to optimum.

3. Pepsin has no inactivating influence on the amylases. On the other hand, some protection against inactivation is noticeable.

4. Similar protection against inactivation at alkaline *p*H ranges is afforded by pancreatin. Marked synergism is also observed in the saccharification of starch by fungal or malt amylases in combination with pancreatic amylase.

5. The significance of the results obtained is discussed in relation to the use of amylase preparations in medicine.

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Action of Dolophine on the Animal Uterus*

By CLARENCE E. POWELL and K. K. CHEN†

Results of a study of the action of Dolophine on the isolated uteri of certain laboratory animals are reported. The action of Dolophine appears to depend on the initial uterine activity. It inhibits those which are in rhythmic movements, but stimulates others which are stationary.

DOLOPHINE (Methadon, Lilly) is a new analgesic of German origin, disclosed after V-J Day by the Technical Industrial Intelligence Committee, U. S. Department of State (1). Its resemblance to morphine and its addiction liabilities were elucidated by Scott and Chen (2), and Isbell, Wikler, Eddy, Wilson, and Moran (3), respectively. The spasmolytic action of Dolophine on the rabbits' and rats' uteri was investigated by Kirchhof, Uchiyama, and David (4, 5).

Although morphine is frequently used in labor, its action on the puerperal uterus is not completely understood. Krueger, Eddy, and Sumwalt (6), who have summarized the information on opium alkaloids, believed that any effects of morphine on the human uterus in labor were mainly the result of the central action. In animals, morphine increases the tone of excised and intact uteri in low concentrations, but decreases the tone in high concentrations (6).

Dolophine has been applied in obstetrics, but was found to cause depression of fetal respiration (7, 8). Notwithstanding, a thorough study of its action on the uteri of different species of animals would be desirable. This prompted the presentation of the present data on the isolated uterus, the uterus *in situ*, and the uterus in spasm induced by stimulating substances. A 0.5–1 per cent solution of Dolophine, freshly prepared, was employed throughout the experiments.

Isolated Uterus.—Uteri of nonpregnant rabbits, rats, golden hamsters, cats, and guinea pigs were removed, and short strips suspended in aerated Ringer's or Tyrode's solution. Their activities were recorded on a smoked drum. After a control period of observation, Dolophine was added to the bath, and the response watched until recovery took place. The rabbits used in this study were pretreated with estrone, because, as shown previously by Wick and Powell (9), this made them more reactive to different drugs.

The results are summarized in Table I. It should be noticed that of 19 strips of rabbits' uteri, 2 showed rhythmic movements while 17 were inactive. Dolophine in various concentrations stimulated the nonrhythmic uteri, but inhibited the uteri in rhyth-

TABLE I.—ACTION OF DOLOPHINE ON ISOLATED UTERUS AND UTERUS *in situ*

	Animal	Condition of Uterus	Dose Range	Number of Observations	Results
Isolated uterus	Rabbit	Nonrhythmic	1:650,000–1:10,000	17	Stimulation
		Rhythmic	1:50,000–1:10,000	2	Inhibition
	Guinea pig	Nonrhythmic	1:1,000,000–1:10,000	7	No action
			1:1,000,000–1:10,000	6	Stimulation
		Rhythmic	1:50,000–1:10,000	2	Inhibition
	Rat	Rhythmic	1:400,000–1:10,000	9	Inhibition
	Hamster	Rhythmic	1:100,000–1:5,000	6	Inhibition
			1:200,000	2	No action
	Cat	Rhythmic	1:200,000–1:10,000	4	Inhibition
Isolated uterus in spasm	Rabbit	Treated with mecholyl	1:100,000–1:7,500	10	Inhibition
		Treated with ergonovine	1:40,000–1:5,000	11	Inhibition
	Guinea pig	Treated with pituitary extract	1:100,000–1:50,000	4	Inhibition
			1:50,000–1:25,000	4	Slight inhibition
Uterus *in situ*	Rabbit	Nonpregnant, rhythmic	0.5–2 mg./Kg.	6	Inhibition
			0.5 mg./Kg.	1	Stimulation
			0.25 mg./Kg.	1	No action
	Cat	Nonpregnant, feebly rhythmic	1.0 mg./Kg.	1	Stimulation
			1.0 mg./Kg.	1	No action
			0.5 mg./Kg.	1	No action
	Dog	*Post partum*, rhythmic	5.0 mg./Kg.	1	Stimulation
			5.0 mg./Kg.	1	Slight inhibition
			3.0 mg./Kg.	1	No action
			2.0 mg./Kg.	1	Slight stimulation

* Received May 27, 1948, from the Lilly Research Laboratories, Indianapolis, Ind.
† We are indebted to Mr. Lester Le Compte, Jr., for his valuable assistance in these experiments.

mic activity. The duration of stimulation lasted four to five minutes and that of inhibition, thirteen to fifteen minutes. Examples of such opposite responses are shown in Fig. 1.

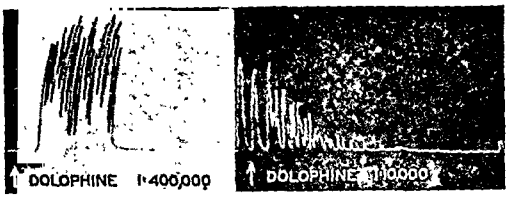

Fig. 1.—Action of Dolophine on isolated rabbits' uteri. Note the stimulation on an inactive strip, and the inhibition of the rhythmic strip.

With guinea pigs, 13 out of 15 strips were inactive at the start, 6 of which reacted to Dolophine by contraction, while 7 remained inactive. The other 2 strips in rhythmic activity relaxed upon the addition of Dolophine. The rat uteri were all in rhythmic activity, and all showed inhibition to Dolophine. All 8 strips of the hamster uteri were in rhythmic activity; 6 of them relaxed and the remaining 2 failed to respond. The inhibitory action of Dolophine in rats and hamsters is well illustrated in Fig. 2. Inhibition was also the rule with the 4 strips of the cat uteri.

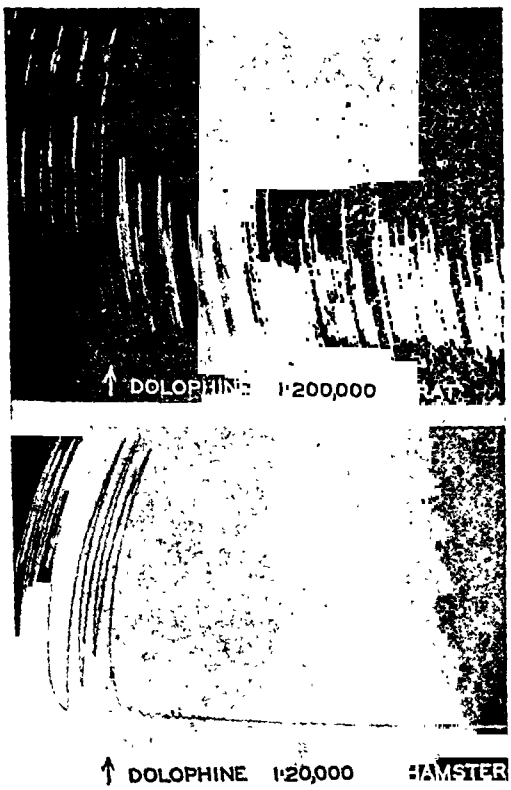

Fig. 2.—Inhibitory action of Dolophine on the isolated rat's and hamster's uteri.

Uterus in situ.—Uterine horns of 8 rabbits and 3 cats were each connected to a writing lever with a string through 2 pulleys—for kymographic recording. The uterine movements of 4 dogs, one to three days *post partum*, were each registered through a balloon inserted into the uterine cavity, and attached to a tambour. All animals were anesthetized with ether. Dolophine in various doses was injected intravenously.

As shown in Table I, 6 of 8 nonpregnant rabbits responded to Dolophine by inhibition—the dose being between 0.5–2 mg. per Kg. An example is given in Fig. 3. One animal showed stimulation of uterine movements with a dose of 0.5 mg. per Kg. The eighth rabbit was without any effect with a dose of 0.25 mg. per Kg.

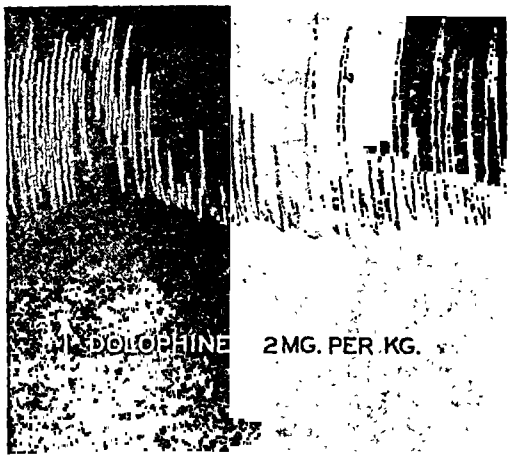

Fig. 3.—Inhibitory action of Dolophine on the rabbit's uterus *in situ*.

Less certain was the action of Dolophine on the cat's uterus *in situ*. One was stimulated with a dose of 1 mg. per Kg., but another failed to respond with the same dose. A dose of 0.5 mg. per Kg. was also without effect on a third animal. It must be mentioned that the initial uterine activity of these animals was feeble.

Similar uncertainty of results was noted on the uterus of *post partum* dogs. Dolophine by slow intravenous injection in the dose of 5 mg. per Kg. caused stimulation in 1 animal, but slight inhibition in another. Small doses were either ineffective or slightly stimulating.

Isolated Uterus in Spasm.—Spasm was induced in isolated rabbits' and guinea pigs' uteri by mecholyl iodide, ergonovine maleate, or pituitary extract. It is obvious from Table I that inhibition uniformly occurred with the isolated rabbit's uterus when Dolophine was added at the peak of action of mecholyl or ergonovine. With the isolated guinea pig's uterus, Dolophine in concentrations of 1 : 100,000–1 : 50,000 released the spasm caused by pituitary extract, but in concentrations of 1 : 50,000–1 : 25,000 it exerted a slight stimulating action, probably indicating a synergistic action with pituitary extract.

DISCUSSION

The action of Dolophine on the uterus appears to depend on the state of its initial activity. If it is in rhythmic movements, the drug causes relaxation; but if it is inactive at the start, the drug usually produces contractions. These opposite effects occur uniformly with the isolated uteri of rabbits, guinea pigs, rats, hamsters, and cats.

As to the uterus *in situ* in etherized rabbits, which shows rhythmic activity, Dolophine is inhibitory in the majority of experiments. The action of the drug on the nonpregnant cat's uterus or the dog's *post partum* uterus is less certain; stimulation of a mild degree seems to be more frequent than slight inhibition. In all the anesthetized animals, the central action may contribute to the modification of the uterine response.

The action of Dolophine is probably not on the autonomic nervous system of the uterus. The lack of unidirectional response would make such a possibility unlikely. It seems that the drug is endowed with a direct muscular action capable of relaxing an active, but stimulating an inactive, uterus. This may also explain the release of spasm by Dolophine induced by mecholyl iodide, ergonovine maleate, or pituitary extract. Only in relatively strong solutions does Dolophine show a slight synergistic action with pituitary extract on the isolated guinea pig's uterus.

SUMMARY

1. The action of Dolophine on the isolated uteri of rabbits, guinea pigs, rats, golden hamsters, and cats appears to depend on the initial uterine activity. It inhibits those which are in rhythmic movements, but stimulates others which are stationary.

2. Dolophine inhibits rabbits' rhythmic uteri *in situ* in the majority of experiments. The results are less certain with cats' uteri which are feebly rhythmic, and dog's *post partum* uteri which are definitely rhythmic.

3. Dolophine releases the spasm of isolated rabbits' uteri, induced by mecholyl or ergonovine. Weak solutions of Dolophine cause relaxation of guinea pigs' uteri in spasm induced by pituitary extract, but relatively stronger solutions may result in a slight stimulation.

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(9) Wick, H. J., and Powell, C. E., THIS JOURNAL, 3 46(1942).

1949 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1949 Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to the Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, Dr. Robert P. Fischelis, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1949 Award, nominations must be received before January 1.

Establishment of the iodine award was first announced at the Milwaukee convention of the AMERICAN PHARMACEUTICAL ASSOCIATION which administers the competition.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During a period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council and which functions under prescribed rules.

Should the award committee fail to find a suitable recipient in any year, two awards may be made during the next year if suitable recipients are selected.

Microcrystallographic Differentiation of *dl*-Amphetamine Sulfate (Benzedrine) and *d*-Amphetamine Sulfate (Dexedrine)*

By GEORGE L. KEENAN†,‡

A microscopic method for quickly and accurately differentiating these two forms of amphetamine sulfate has been devised, applying the immersion method and microchemical test with a suitable reagent. The *d*- and *dl*-forms were found to be markedly different in respect to their optical crystallographic properties as determined by the polarizing microscope. Crystalline addition products formed with gold chloride reagent showed that the microchemical test was equally as valuable for a rapid and accurate diagnosis.

THERE appears to be no record of a microscopic study of the *dl*- and *d*-forms of amphetamine sulfate. More recently Haley (2) described a chemical differentiation of several sympathomimetic amines, *dl*- and *d*-amphetamine sulfate being included among them. The occasion arose for a rapid and accurate method for distinguishing these two forms microscopically, applying both optical and strictly microchemical techniques. Both substances lend themselves to a study of this nature, the results being so striking as to justify calling them to the attention of the analyst.

dl-Amphetamine Sulfate (Benzedrine).—In ordinary light, the microscopic examination shows that the substance is definitely crystalline, consisting largely of small, irregular particles, frequently elongated but lacking in any significant geometric forms. In this respect, this form is not as satisfactory for microscopic study as the *d*-compound. Crossed nicols revealed nothing significant and the fragments were too small to reveal interference figures by the conoscopic method. From the standpoint of the refractive indices, however, one value, the minimum index (α), is so frequently found lengthwise (oriented parallel to the vibration plane of the lower nicol) as to be very valuable in diagnosis. This index, $\alpha = 1.520$, is the only one measurable, the unsatisfactory cleavage precluding additional determinations that would be trustworthy.

Attention has already been called to the crystalline addition product formed with gold chloride reagent (1). The very thin, yellow, quadratic plates are quite striking when examined microscopically in ordinary light, frequently so thin as to exhibit faint interference colors. Many of the plates extinguish sharply when examined with crossed nicols (parallel polarized light) and revolution of the microscope stage, others remaining bright under the same conditions. The latter show a distinct biaxial interference figure by the conoscopic examination, two isogyres of the figure appearing in the field of view. The axial angle is small with negative optic sign.

d-Amphetamine Sulfate (Dexedrine).—Unlike the racemic compound, this form, when examined microscopically in ordinary light, exhibits a platelike habit, many of the plates being 6–8-sided in outline, some elongated, usually lying on the broad face, occasionally tipped on edge and simulating the appearance of needles. With crossed nicols, many of the plates do not extinguish sharply when the microscope stage is revolved. The sign of elongation is negative. With the conoscopic examination, biaxial interference figures showing the axial plane frequently are observed, 2E being small and the optic sign positive. The refractive indices (applying the immersion method) are: $\alpha = 1.505$, $\beta = 1.545$, $\gamma = 1.608$, all ± 0.002. α and β usually found on plates lying on the broad face and α and γ on plates tipped on edge. The α and β values are most conveniently found and diagnostic for the substance.

Gold chloride reagent (5%) also forms a crystalline addition product with the *d*-compound but of a significantly different habit than that characteristic of the racemic form. Both compounds are readily soluble in the reagent, but in the case of the *d*-form, the crystalline addition product forms almost immediately, crystallizing out in the form of short, stout, prismatic rods and needles (parallel extinction with crossed nicols), and not quadratic plates as in the case of the *dl*-form. Furthermore, the plates of the racemic compound come out much more slowly, it frequently being necessary to add an additional amount to the reagent drop and scratch the slide with the rod. The rate of formation of the respective addition products with gold chloride is quite as significant as the marked difference in their habit.

SUMMARY

The *d*- and *dl*-forms of amphetamine sulfate exhibit marked differences microscopically, in respect to both the optical crystallographic properties and the difference in the habit and rate of formation of the crystalline addition products with gold chloride reagent. By these two

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Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
† The author wishes to acknowledge the valuable assistance of Dr. Thomas J. Haley, Los Angeles, Calif.; Smith, Kline & French Laboratories, Philadelphia; and Dr. Justin L. Powers, Editor of THIS JOURNAL. Acknowledgment is also due the Chemistry Department, Baldwin-Wallace College, Berea, Ohio, for use of apparatus.
‡ Present address: 165 Westwood Drive, Strongsville, Ohio.

TABLE I.—MICROSCOPIC DIFFERENCES OF *d*- AND *dl*-AMPHETAMINE

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dl-Amphetamine SO_4 Gold salt	Irregular fragments	1.520	...	...
	Quadratic plates			
d-Amphetamine SO_4 Gold salt	Plates, rods	1.505	1.545	1.608
	Rods			

methods both compounds can be quickly and accurately differentiated.

Table I summarizes the significant microscopic differences.

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(2) Haley, Thomas J., THIS JOURNAL 36, 301(1947).

A Study of Phosphorus Uptake in the Rat Tooth by Radioactive Tracer Techniques*

By CATHERINE J. HURLEY and JOHN E. CHRISTIAN†

The results of a study to determine the rate of uptake of phosphate in rats' teeth following the intraperitoneal injection of potassium biphosphate containing radioactive phosphorus are reported. The techniques employed are described and the results obtained are discussed and tabulated.

A STUDY of phosphorus uptake in the rat tooth by radioactive tracer technique was considered necessary in view of subsequent experiments which were to be carried out using rat teeth which contained radioactive phosphorus. Such experiments required that the rat teeth be made sufficiently radioactive with a minimum amount of radioactive phosphate solution and in as short a time as possible.

A complete survey of the literature concerning phosphorus uptake in teeth using radioactive tracers indicates that no data have been reported on the rate of phosphorus uptake when the radioactive phosphate solution is injected intraperitoneally. The intraperitonael injection of radioactive materials is considered by the authors to be the most efficient method available for this type of work since this method is rapid, complete dosage is assured, and rapid absorption results. In addition, the possibility of radioactive contamination is less by this method.

LITERATURE REVIEW

Greenberg (1) described the work of Chievitz and Hevesy, who added phosphorus to food and demonstrated that an appreciable part found its way to the bones, teeth, muscle, and other organs.

Hevesy, Holst, and Krogh (2) found that in the rat the labeled phosphorus was deposited in close proximity to the pulp from which it is derived and that it was equally distributed throughout the incisors.

Hevesy (3) developed a technique to avoid corrections for the difference in the absorption of the beta rays emitted by samples of different thickness and composition.

Hevesy, Levi, and Rebbe (4) injected radioactive phosphorus subcutaneously into rabbits. In fifty days, the incisor dentin phosphorus of the apical and medial parts was almost completely replaced.

Hevesy and Armstrong (5) injected radioactive phosphate subcutaneously into cats, removed the teeth, and separated the enamel. They found there was a slow exchange of phosphorus but no ability of the enamel to undergo significant changes after eruption as a result of nutritive alterations.

Armstrong (6) agitated tooth samples in radioactive phosphate solutions to determine the rate of exchange of phosphate.

Armstrong and Barnum (7) separated the dentin from the enamel of cats that had been injected subcutaneously with radioactive sodium phosphate. They found that the enamel exchanged phosphate at a rate approximately 5% that of dentin.

Armstrong (8) also calculated the relative uptakes of phosphorus and calcium in the incisor dentin and enamel and the molar dentin and enamel.

LeFevre and Bale (9) measured the amount of marked phosphorus deposited in the teeth of rats killed four to twenty hours after having been fed radioactive phosphorus.

Manly and Bale (10) also determined the phosphorus turnover in the inorganic portion of rat teeth.

Manly, Hodge, and Van Voorhis (11) cut grooves in the teeth of a dog which had been given radioactive phosphorus by stomach tube. There was no difference in the phosphorus content of the scarred and unscarred teeth.

* Received March 3, 1948, from the Research Laboratories, Purdue University, School of Pharmacy, Lafayette, Ind.
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Johannson, Falkenheim, and Hodge (12) studied the relative abilities of powdered dentin and enamel to adsorb ions from aqueous solutions. It was shown that for short periods the dentin adsorbed more than the enamel.

In a further study, Hodge and Falkenheim (13) found that for longer periods there was a complete reversal of the order of adsorption: the enamel adsorbed more than the dentin.

Hodge, *et al.* (14), determined the amount of phosphate adsorbed by calcified tissues from the KOH-ethylene glycol solution used to extract the organic portion of teeth.

Sognnaes and Volker (15) and (16) worked with cats, dogs, and a monkey. They separated the enamel and the dentin of the teeth and determined the concentration of phosphate. The concentration in the dentin was inversely proportional to the density while the opposite seemed to hold for the enamel.

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Using X-ray film, McCauley and Gilda (20) determined the absorption of phosphate by the pulpless teeth of dogs. There was a striking decrease in the amount of labeled phosphorus in pulpless teeth.

The foregoing is a complete review of the literature that concerns work on the teeth using radioactive phosphorus.

EXPERIMENTAL

Since no data are available in the literature concerning the rate of uptake of phosphorus in rats' teeth on intraperitoneal injection of radioactive phosphate and since the amounts of active phosphate necessary to confer sufficient activity to the teeth for experimental study has not been indicated, the following experiments were designed to provide such information.

Experiment 1: Each of a group of 120 white rats weighing approximately 250 Gm. was injected intraperitoneally with 1 cc. of a solution containing 0.1 Gm. per cc. of radioactive potassium biphosphate (KH_2PO_4) with an activity of 200,000 counts per minute per cc. as measured in a cup 15 mm. in diameter 1.3 cm. from an Eck and Krebs counter tube. At the end of varying periods of time 15 rats were killed and the activity of the teeth determined in the following manner: From all rats two teeth were removed after boiling the heads in water for five minutes to aid removal. The teeth were washed with 0.5% potassium hydroxide and water, and dried; the teeth were then ground to a fine powder, and a quantity weighed into a standard counting cup 15 mm. in diameter. Two drops of 20% sulfuric acid were added and the residue dried in an oven at 140° after which it was ashed in a muffle furnace at 550° to 600°. The activity of the residue was then determined by a Geiger-Muller counter. All counts were corrected to a standard time. The results of the rate of phosphorus uptake are given in Table I.

TABLE I.—RATE OF PHOSPHORUS UPTAKE IN RATS' TEETH ON INTRAPERITONEAL INJECTION OF PHOSPHATE

Rat No.	Time Killed After Injection, Hr.	Average Activity per 100 Mg. of Tooth, C. p. m.[a]
1–15	1/2	52.6
16–30	1	55.9
31–45	2	72.1
46–60	5	88.1
61–75	10	101.0
76–90	20	287.5
91–105	53	98.2
106–120	66	108.3

[a] C. p. m. = counts per minute.

These results indicate that the phosphorus after a single intraperitoneal injection of phosphate is taken up in the teeth quite rapidly in the first twenty hours, after which the activity of the teeth diminishes.

Experiment 2: This experiment was designed to determine the activity of radioactive potassium biphosphate necessary to produce a desirable activity in the rats' teeth for experimental purposes in the study of mineral depletion. A group of 160 white rats, weighing about 250 Gm., was injected with 1 cc. of a solution containing 0.1 Gm. of radioactive potassium biphosphate of varying radioactivity. All rats were killed at the end of twenty hours and the activity of the teeth determined as in the preceding experiment. Table II gives the results obtained.

TABLE II.—THE ACTIVITY OF RATS' TEETH AFTER INTRAPERITONEAL INJECTION OF PHOSPHATE SOLUTION OF VARYING RADIOACTIVITY

Rat No.	Activity of Phosphate, C. p. m.[a] per Ml.	Average Activity per 100 Mg. of Tooth C. p. m.[a]
1–20	233,412	629
21–40	438,664	761
41–60	594,823	782
61–80	854,238	984
81–100	1,329,425	1156
101–120	1,998,511	1739
121–140	1,318,216	3265
141–160	6,274,742	4882

[a] C. p. m. = counts per minute.

These data show conclusively that the activity of rats' teeth after injection intraperitoneally of radioactive phosphate is a linear function of the activity of the phosphate solution injected.

SUMMARY

The purpose of this study was to determine the rate of uptake of phosphate in rats' teeth on intraperitoneal injection of phosphate and to determine the phosphate activity necessary to incorporate sufficient activity in the teeth for phosphorus depletion studies. The results indicate

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61–75	10	101.0
76–90	20	287.5
91–105	53	98.2
106–120	66	108.3

[a] C. p. m. = counts per minute.

These results indicate that the phosphorus after a single intraperitoneal injection of phosphate is taken up in the teeth quite rapidly in the first twenty hours, after which the activity of the teeth diminishes.

Experiment 2: This experiment was designed to determine the activity of radioactive potassium biphosphate necessary to produce a desirable activity in the rats' teeth for experimental purposes in the study of mineral depletion. A group of 160 white rats, weighing about 250 Gm., was injected with 1 cc. of a solution containing 0.1 Gm. of radioactive potassium biphosphate of varying radioactivity. All rats were killed at the end of twenty hours and the activity of the teeth determined as in the preceding experiment. Table II gives the results obtained.

TABLE II.—THE ACTIVITY OF RATS' TEETH AFTER INTRAPERITONEAL INJECTION OF PHOSPHATE SOLUTION OF VARYING RADIOACTIVITY

Rat No.	Activity of Phosphate, C. p. m.[a] per Ml.	Average Activity per 100 Mg. of Tooth C. p. m.[a]
1–20	233,412	629
21–40	438,664	761
41–60	594,823	782
61–80	854,238	984
81–100	1,329,425	1156
101–120	1,998,511	1739
121–140	1,318,216	3265
141–160	6,274,742	4882

[a] C. p. m. = counts per minute.

These data show conclusively that the activity of rats' teeth after injection intraperitoneally of radioactive phosphate is a linear function of the activity of the phosphate solution injected.

SUMMARY

The purpose of this study was to determine the rate of uptake of phosphate in rats' teeth on intraperitoneal injection of phosphate and to determine the phosphate activity necessary to incorporate sufficient activity in the teeth for phosphorus depletion studies. The results indicate

that the most economical, rapid, and efficient method for obtaining rat teeth containing sufficient radioactive phosphorus to insure at least 35 counts per minute per mg. (as measured in a 15-mm. cup 1.5 cm. from an Eck and Krebs counter tube) is to inject 250-Gm. rats intraperitoneally with 1 cc. of a radioactive potassium biphosphate solution containing 0.1 mg. per cc. and having an activity of between five and seven million counts per minute per cc., and remove the teeth twenty hours after injection. Teeth containing an activity of at least 35 counts per minute per mg. have been shown to be satisfactory for studies involving the phosphorus depletion of teeth.

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Book Reviews

Clinical Toxicology. 2nd ed. By CLINTON H. THIENES and THOMAS J. HALEY. Lea and Febiger, Philadelphia, 1948. 373 pp. 13 x 20 cm. Price $4.75.

The modern approach to toxicology is an experimental one and this has led to many practical, life-saving benefits. Dr. Thienes' book reflects this approach and consequently it is essentially factual. Several points brought out are suggestive of further research needed. For example, in view of the biphasic character of morphine action on the central nervous system one wonders about the advisability of using caffeine to overcome respiratory depression.

This revision of *Clinical Toxicology* introduces a wealth of new material on such things as DDT, thiouracil, BAL, streptomycin, and dicumarol and includes uses of proteins, amino acids, vitamins, etc., in treatment of poisoning. The section on chemical identity of poisons has been enlarged also and new methods for preserving tissues after autopsy have been introduced.

If pharmacists find it impossible to have more than one book in this field, this one should be given highest consideration for the book of choice.

Hemostatic Agents with Particular Reference to Thrombin, Fibrinogen, and Absorbable Cellulose. By WALTER H. SEEGERS and ELWOOD A. SHARP. Charles C. Thomas, Springfield, Illinois, 1948. xii + 131 pp. 15 x 23 cm. Price $4.50.

The mechanism of the clotting of blood has never been completely understood, a fact which led to complex theories not always easy to comprehend. The authors of this book have given a very clear accoun of this physiological mechanism.

Although it was just ten years ago that Seegers Warner, Brinkhous, and Smith first used thrombi successfully as a hemostatic agent in animals, it i frequently used in surgery today. Its use is dis cussed briefly in this stimulating book, as is also th use of fibrinogen. Modern application of such prod ucts as oxidized cellulose, fibrin foam, and gelati sponge is covered also.

All of the many complex problems of research and development of hemostatic agents have not bee solved. Such a carefully documented review (37 references) will be helpful to those interested in fol lowing the important developments in this area.

Life, Its Nature and Origin. By JEROME ALEXANDER. Reinhold Publishing Company, New York, 1948. viii + 291 pp. 14 x 23 cm. Price $5.00.

It was in the spring of 1899 when young Jerome Alexander submitted a thesis for his master's degree entitled "The Importance and Trend of Recent Work on the Chemistry of Life and the Products of Life." During all of the intervening years Dr. Alexander has been keenly interested in this subject although his major professional career has been in the colloid field. The author has integrated a vast amount of scientific literature into a picture of biocatalysis that probably accounts for as accurate an account of the physical origin of life as is possible with today's knowledge.

Those interested in a philosophic interpretation of science will find Dr. Alexander's book uplifting and stimulating.

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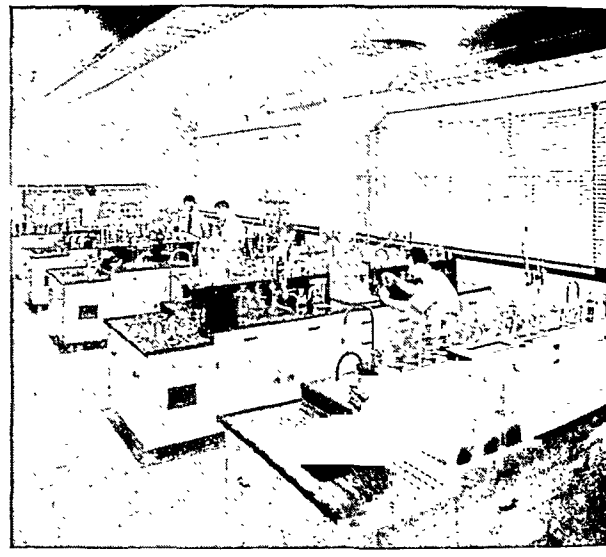

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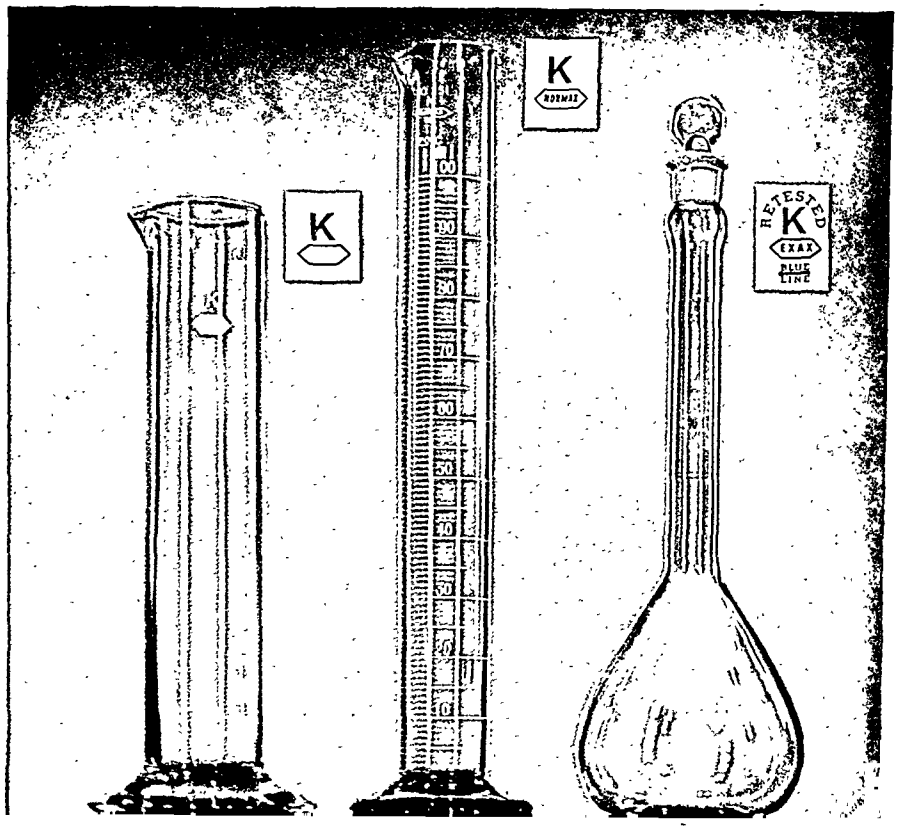

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SCIENTIFIC EDITION

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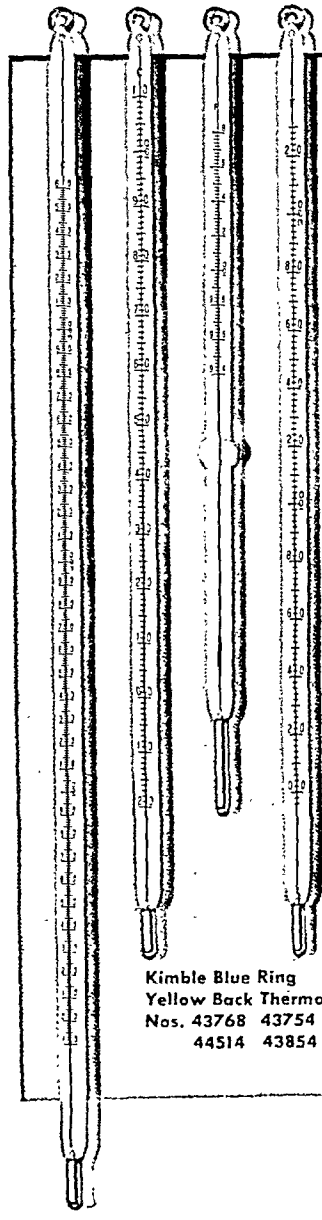

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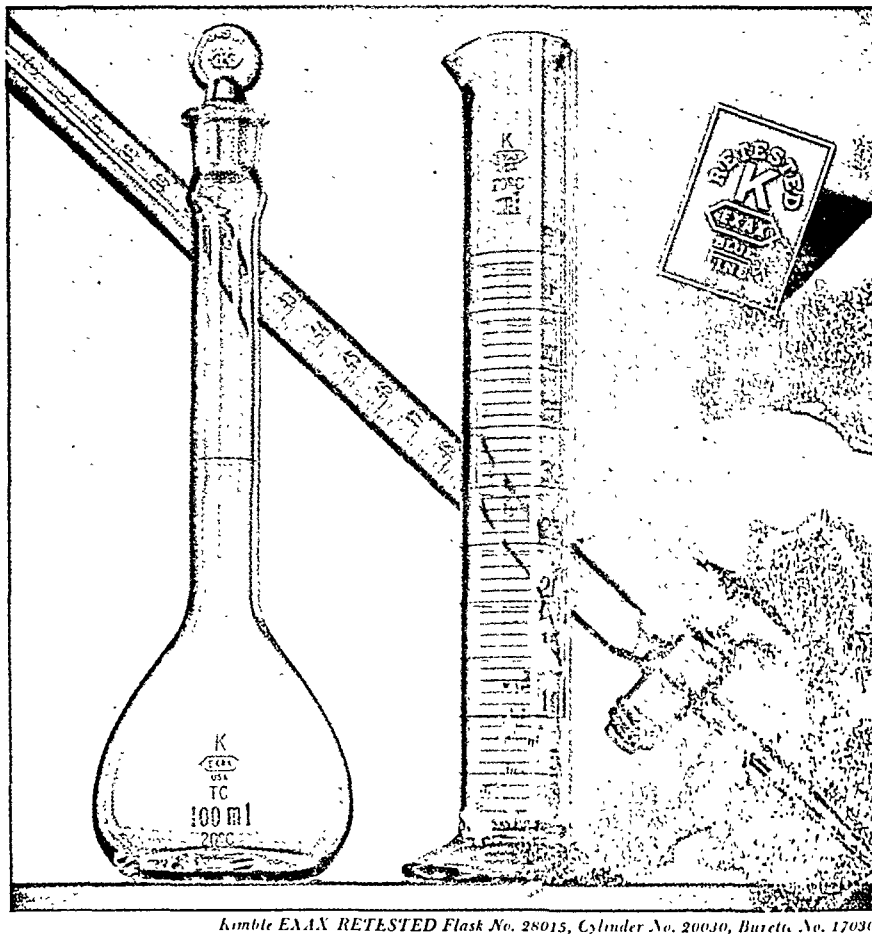

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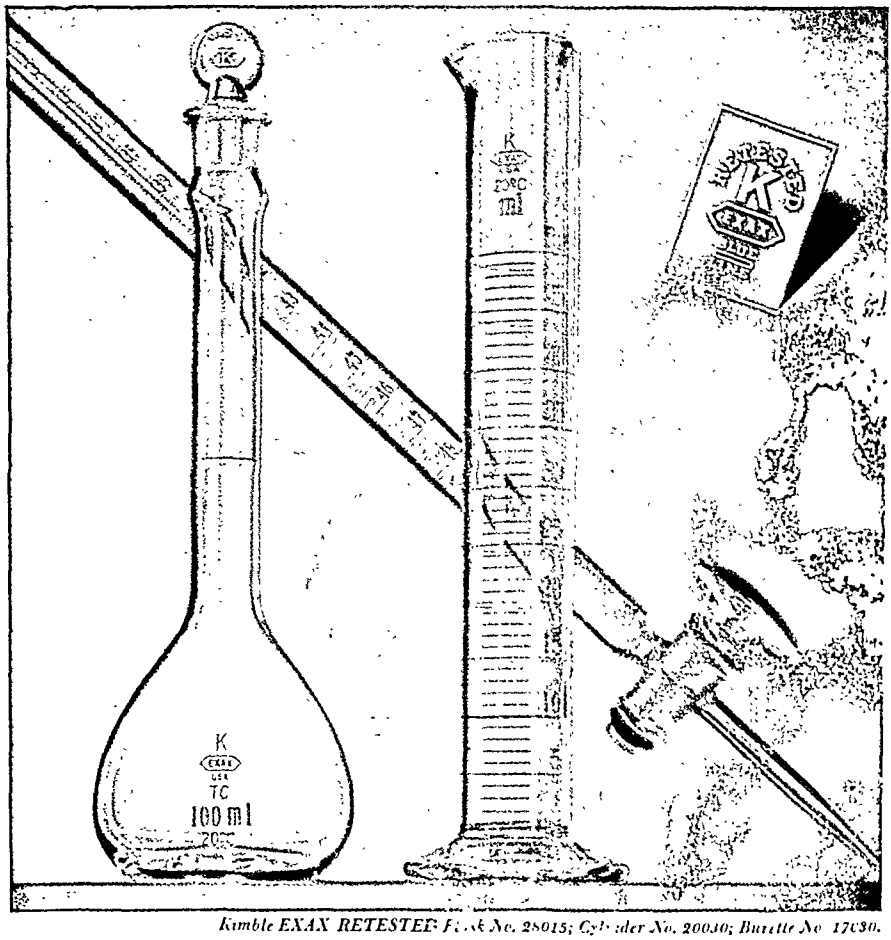

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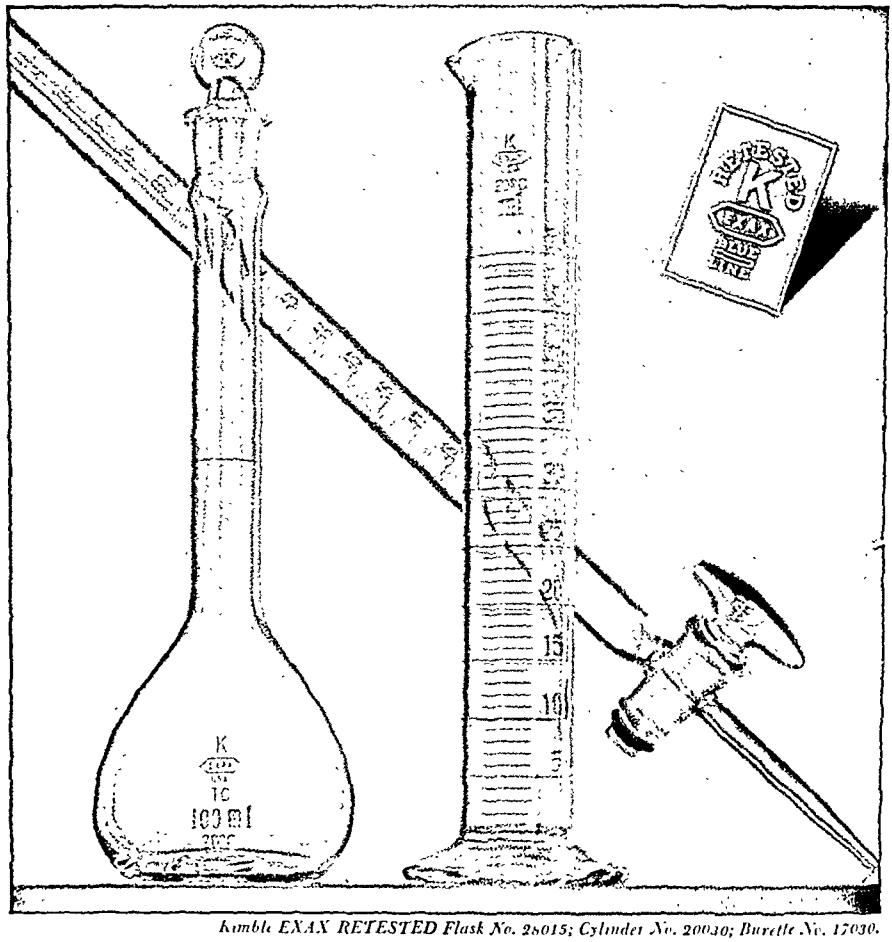

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Evaluation of Hydrophilic Properties of Bulk Laxatives Including the New Agent, Sodium Carboxymethylcellulose*

By RUDOLPH H. BLYTHE, JOHN J. GULESICH, and HARLAN L. TUTHILL

This study was undertaken to find a laxative agent superior to those now in use. The hydrophilic properties of standard bulk laxatives are compared with two new synthetic agents. New and modified methods for the *in vitro* evaluation of such agents are described.

THE NATURAL gums have recognized faults when used as bulk laxatives. Clinically, they may cause antigenic reactions (1, 2) and lead to obstructions (1, 2) or a feeling of flatulence (3). Pharmaceutically, different batches show variation in gel strength and cleanliness. It was evident that a synthetic hydrophilic substance would eliminate the pharmaceutical variables and might be superior clinically. The cellulose derivatives, methylcellulose (MC)[1] (4) and sodium carboxymethylcellulose (CMC)[2] (5, 6), are strongly hydrophilic, resistant to digestion and nontoxic. They were, therefore, compared with various vegetable colloids.

The *in vitro* tests (1, 3) heretofore used to evaluate laxatives are only distantly related to conditions in the gastrointestinal tract. Gray and Tainter (1) supplemented their laboratory tests with clinical data on wet and dry stool weights. In the present study, however, work with the stool weight method, although attempted, was discontinued when it was found that statistically significant data could not be obtained under conditions practical for the investigation. It appeared that it would be necessary to place a very large number of institutionalized people on a standard diet for a considerable period of time. Since this would create an artificial condition, it seemed preferable to develop a physicochemical measurement that would bear a similarity to the physiological process and eliminate biological variabilities.

* Received August 9, 1948, from Smith, Kline & French Laboratories, Philadelphia, Pa.
Presented to the Subsection on Pharmacy of the American Association for the Advancement of Science at its Annual Meeting, Chicago, Ill., Dec. 27, 1947.
The authors wish to thank Edmund W. Wydrzynski and Catherine F. DeFonso for their technical assistance in this investigation.

[1] Methylcellulose High Viscosity, Dow Chemical Co.

[2] Sodium Carboxymethylcellulose High Viscosity, Hercules Powder Co.

Three properties are significant in the *in vitro* evaluation of bulk laxatives: (*a*) the volume of water absorbed in various media (water and artificial gastric and intestinal juices), (*b*) the viscosity and texture of the gel formed, and (*c*) the ability of the gel to retain water.

To avoid causing gastric discomfort, the agent should swell in the alkaline intestinal tract but not in the acid gastric juice. The physical characteristics of the gel determine its ability to mix with the intestinal contents; a high degree of miscibility may be the primary factor in avoiding the formation of gummy impactions in the gut. In order to keep the intestinal contents soft throughout the tract, the gel must be able to retain its water against pull of osmotic and other water-absorbing forces within the intestinal wall.

EXPERIMENTAL

Method I. Gel Volume and Liquid Absorption.—Add 5 Gm. of the hydrophilic material to 500 ml. of liquid (water or artificial gastric juice or artificial intestinal juice) in a 500-ml. graduated cylinder. Agitate contents gently by inverting every fifteen minutes for two hours; then let stand undisturbed for eighteen hours. Note the volume to which the gel has swollen (Gel Volume) and pour the material onto a moistened, fine glass wool filter ($3^1/_2$ inches square and $^1/_4$ inch thick, weighing 2 Gm.) held in a 6-inch glass funnel. Allow the liquid to drain for five minutes into a second 500-ml. graduate. Calculate by difference the volume of liquid retained by the gel (Liquid Absorption). As a variation of the above procedure, a single layer of unwashed absorbent cheesecloth (Grade 80) is used as the filter medium in place of the glass wool.

FORMULA FOR ARTIFICIAL GASTRIC JUICE

Hydrochloric Acid	0.60%[3]
Pepsin	0.05%

FORMULA FOR ARTIFICIAL INTESTINAL JUICE

Sodium Bicarbonate	1.50%
Bile Salts (Bacto)	1.00%
Pancreatin	0.28%

Method II. Gel Volume with Increased Agitation.—Proceed as in Method I but omit manual inversion and place test mixture in a quart bottle and rotate end over end mechanically at 36 r. p. m. for four hours, pour contents into 500-ml. graduate and allow to stand eighteen hours before reading.

Method III. Water Retention against Osmotic Pull.—The apparatus, as shown in Figs. 1 and 2, is of our own design. The method is based upon the theory that the laxative efficacy of a hydrophilic colloid is proportional to its ability to hold water against absorption from the intestinal tract into the blood stream. For comparative purposes, such efficacy can be quantitatively determined by measuring the ability of a sol or gel to hold water against the osmotic pull of a hypertonic solution when the two media are separated by a semipermeable membrane. As standard conditions of test, we selected a 170-mm. column of a 30% aqueous solution of "Carbowax" 4000W[4] as the hypertonic solution and separated it from a 2% concentration of the hydrophilic colloid being tested by a 600A PT cellophane membrane having an effective diameter of 50 mm.

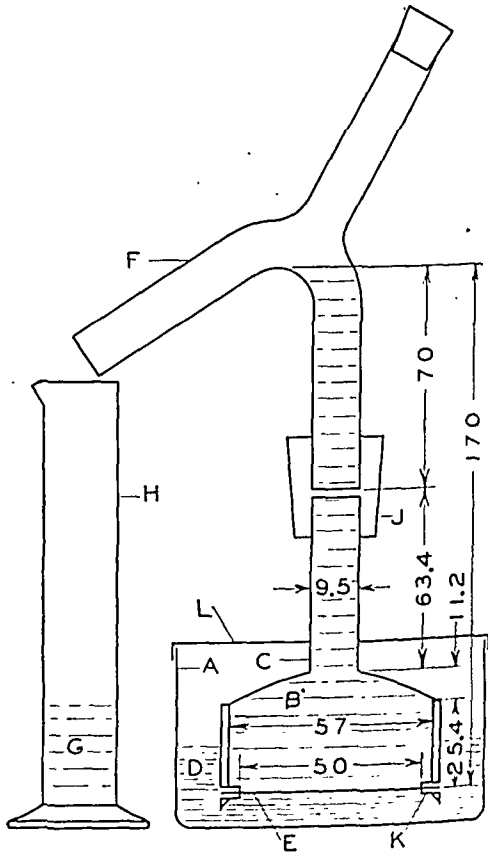

Fig. 1.—Apparatus for determination of water retention against osmotic pull (dimensions in mm.).

A, 90-mm. crystallizing dish; *B*, hypertonic solution; *C*, brass osmometer; *D*, test mixture, 2%; *E*, 600A. PT cellophane (Du Pont) 58 mm. diameter; *F*, 10-mm "Y" tube. Length of arm, 70 mm.; *G*, overflow from osmometer; *H*, 25-ml. graduate; *J*, rubber stopper connection; *K*, rubber washers (Ball No. 10, top seal jar rubbers) held in place with a metal ring and clamps; *L*, "Parafilm" membrane (Menasha Prod. Co., Menasha, Wis.).

[3] Based on Bodansky, Meyer, "Introduction to Physiological Chemistry," ed. 4, John Wiley and Sons, New York, 1938, p. 169. This reference indicates that the concentration of hydrochloric acid as it is secreted is approximately 0.17 *N* or about 0.62%, and the *p*H is approximately 0.88; the *p*H of the formula above is 1.0. The *p*H is an arbitrary one used for convenience, since test conditions prohibit making allowances for differences that occur among persons with varying stomach content or the continuing supply of fresh gastric juice.

[4] Brand of polyethylene glycol obtained from Carbide & Carbon Chemicals Corp.

The test is carried out as follows: Prepare the test mixture by adding 2 Gm. of the hydrophilic material to 98 Gm. of artificial intestinal juice and mix occasionally over a two-hour period. Allow the mixture to stand for eighteen hours so that the hydrophilic substance may become hydrated. Pour this mixture into the glass crystallizing dish (*A*) (see Fig. 1). Pour the hypertonic solution (*B*) into the osmometer (*C*) until it rises in the neck. Then place the osmometer in the test mixture (*D*). Exercise care to avoid trapping air bubbles under the cellophane membrane (*E*). Now fill the osmometer with the "hypertonic solution" until it starts to drop out at the lower, open end of the "Y" tube (*F*). Place a "Parafilm" or "Pliofilm" membrane over the crystallizing dish to reduce evaporation of the hydrophilic gel. Collect the overflow (*G*) (mixture of the "hypertonic solution" and liquid pulled from the hydrophilic gel through the membrane) in 25-ml. graduated cylinders (*H*). Record the volume of liquid obtained over a period of forty-eight hours. Figure 2 is a photograph of the above described setup.

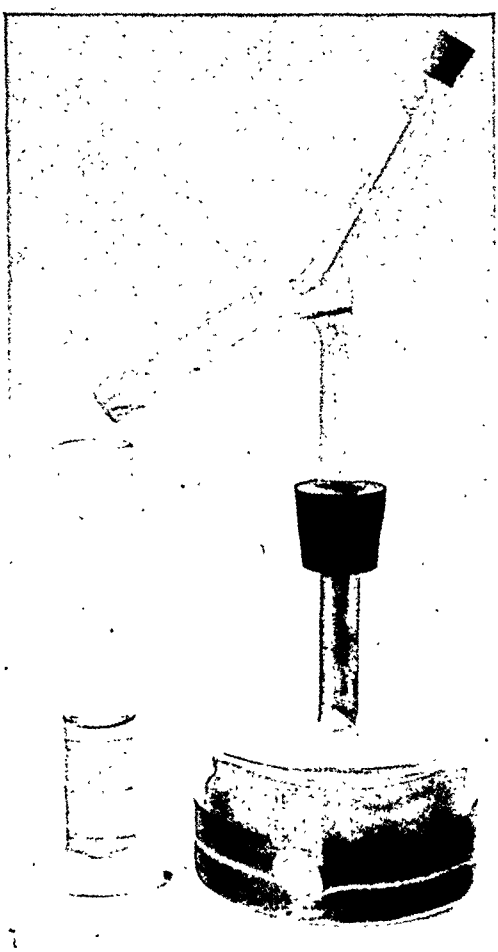

Fig. 2.—Apparatus for determination of water retention against osmotic pull.

DISCUSSION

Effect of the Media on Gel Volume (Table I, Figs. 3 and 4).—The influence of the medium on gel volume is shown in Table I and Fig. 3 (illustrating results with Method I) and Fig. 4 (Method II). All the substances show marked differences in behavior. As has already been observed (1) the karaya and psyllium type substances swell much better in water than in artificial physiological fluids. In the simulated gastric juice they attain, roughly, half the volume obtained in water, and in artificial intestinal juice they swell more than in the acidic medium but less than in water. This is true whether Method I, involving only eight inversions at fifteen-minute intervals, or Method II, continuous mild agitation over a four-hour period, is used.

The cellulose derivatives, on the other hand, dissolve equally well in water or in artificial intestinal fluid. They show an important difference in behavior, however, in the simulated gastric juice. When either Method I or Method II is used, sodium carboxymethylcellulose is converted to the free acid which is insoluble; but methylcellulose becomes partly swollen and floats on the surface of the "gastric" medium when Method I is used, and with Method II it goes into complete solution in all three media.

The fact that sodium carboxymethylcellulose does not swell in artificial gastric juice, even when agitated to a degree which reasonably approximates conditions *in vivo*, suggests a clinical advantage. Unlike many bulk laxatives, it should not produce an uncomfortable feeling of fullness. Its insolubility in the artificial gastric juice does not interfere with its efficacy since, upon removal from the "gastric" to the "intestinal" medium, sodium carboxymethylcellulose is reconverted to the soluble sodium salt and readily dissolves.

Viscosity and Texture of the Gel (Table I, Fig. 3).—The cellulose derivatives form a clear viscous solution. Karaya products swell in the various liquids to form gels which retain their granular texture. Psyllium products may be classed between these two extremes. Whole psyllium seed forms gelatinous masses with the inner part of the seed retaining much of its form. The hemicellulose materials derived from psyllium give viscous, pourable, opaque gels with widely varying amounts of insoluble seed material remaining, depending on the brand of product. The character of the gel formed by each test material is shown in Table I.

These characteristics may have important clinical significance. In the first place, a solution should be fully miscible with the intestinal contents and not remain as the gelatinous masses mentioned by Ivy and Isaacs in their study of a karaya product (3). This expectation was substantiated on examination of stools of mice fed sodium carboxymethylcellulose (7). Secondly, the unique ability of the cellulose derivatives to pass through the very small openings of the cheesecloth (Fig. 3) suggests that they are less likely than the gums to form an impaction in a partially obstructed intestine.

Retention of Water (Fig. 5).—Some indication of hydrophilic capacity of certain test substances is obtained with Method I. However, the physical differences between the synthetics and the natural products limit the significance of this test when it is

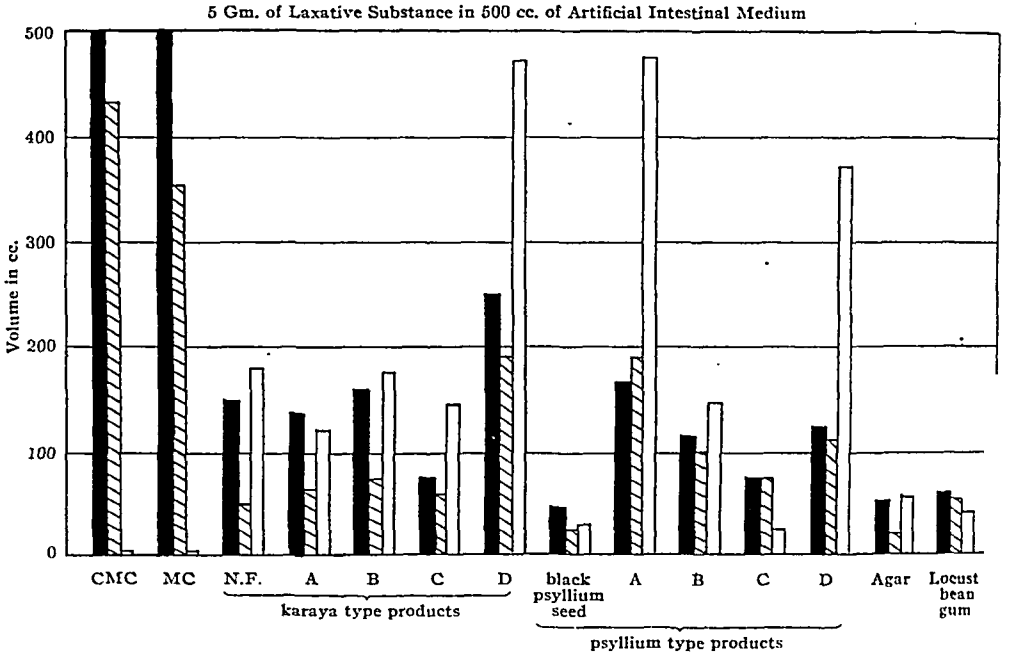

Fig. 3.—Gel volume and liquid absorption (method I). 5 Gm. of laxative substance in 500 cc. of artificial intestinal medium. Key: black, gel volume; shaded, liquid retained on glass wool; white, liquid retained on cheesecloth. Substances indicated above as karaya type products and psyllium type products A to D, inclusive, represent leading commercial products available under various trade names.

applied to dissimilar substances. Method III, devised to minimize the influence of these conditioning factors and to approximate *in vivo* conditions, gives results which seem more significant. As shown in Fig. 5, the cellulose derivatives are clearly superior in hydrophilic properties; the relative efficacy of the various karaya and psyllium preparations differs widely with the source and brand of the product tested.

NEW "HYDROPHILIC" TEST

The factors which limit the value of Methods I and II are viscosity and nature of the gelatinous mixtures formed, differing solubilities in vari media, type of filter used, and degree of agitati A search was therefore made for a way to minin these differences. Ivy and Isaacs (3) were proba attempting this when they compared water-hold properties by measuring resistance to drying o sodium hydroxide in a vacuum desiccator. seemed to us, however, that resistance to loss water through a membrane under the influence c hypertonic solution might not only solve th problems but also give a reasonable indication of ability of the gel to retain water against osmotic pull of the body fluids within the intestinal wall.

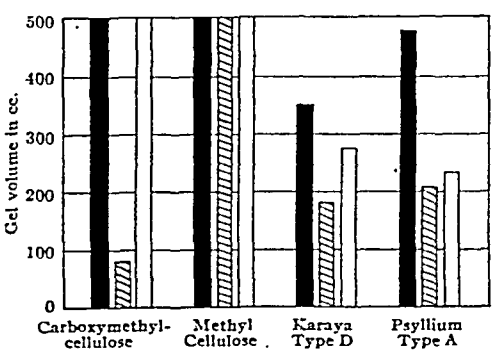

Fig. 4.—Gel volume with increased agitation (method II). Key: black, water; shaded, artificial gastric juice; white, artificial intestinal juice.

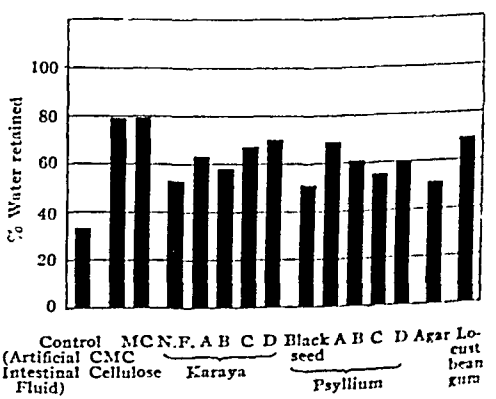

Fig. 5.—Water-holding capacity—against osmotic pull (method III). Forty-eight hour reading.

TABLE I.—GEL VOLUME AND LIQUID ABSORPTION

	Water			Artificial Gastric Juice		Artificial Intestinal Juice		
	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Consistency of Material on Filter	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Liquid Retained on Cheese Cloth, ml.
I. Cellulose Type								
CMC	500	470	Viscous sol	[a]	40	500	430	0
MC	[b, c]	[c]	[c]	[b, c]	[c]	500	355	0
II. Karaya Type								
Karaya, N. F.	205	80	Stiff and lumpy	95	35	150	50	180
A	195	90	Stiff, granular gel	95	40	135	65	120
B	270	140	Stiff, granular gel	115	80	160	75	175
C	180	120	Stiff, granular gel	60	40	75	60	145
D	350	230	Viscous, granular gel	165	125	250	190	470[d]
III. Psyllium Type								
Psyllium Seed Black	55	25	Stiff gel	30	10	45	25	30
A	370	310	Slightly viscous	180	175	165	190	475[d]
B	125	110	Slightly viscous	100	95	115	100	145
C	90	65	Slightly viscous	80		75	75	25
D	175	165	Watery	105	90	125	110	370[d]
IV. Agar	60	45	Soft gel	40	20	50	20	55
V. Locust Bean Gum	133	155	Watery	75	80	60	55	40

[a] Carboxymethylcellulose is converted to the free acid, which is insoluble.
[b] Material floats on surface of liquid in a partially swollen condition.
[c] Does not go completely into solution.
[d] Gum forms film on cheesecloth that prevents unbound liquid from draining.

Method III was, therefore, devised. As shown in Fig. 1, the gel or sol was separated by a cellophane membrane from a hypertonic solution, and the loss of liquid from the gel or sol was measured by the overflow from the osmometer. The remarkably superior water-holding capacity of the synthetic cellulose derivatives is evident from the data in Fig. 5. For comparative purposes, observations were made at forty-eight-hour intervals since, as shown in Fig. 6, the more effective substances approach an equilibrium at that time. Thus longer periods of observation (e.g., one-hundred-twenty hours) were unnecessary.

The use of a polyethylene glycol as a hypertonic agent deserves some comment. This substance was chosen because its large molecular size minimizes its passage through the cellophane membrane. Tests with the 30% solution indicated that after seventy-two hours not more than 1.1% of the polyethylene glycol has passed through the membrane into distilled water on the other side. This concentration makes significant readings obtainable within a relatively short time. The tonicity of 30% solution was calculated as 5.3 on the basis of a freezing-point depression of 2.96° C.

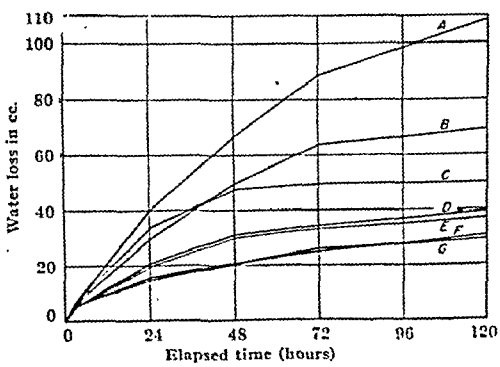

Fig. 6.—Water-holding capacity—against osmotic pull. Water loss rate. Key to curves: *A*, artificial intestinal juice[1] (control); *B*, psyllium seed black; *C*, karaya; *D*, psyllium type A; *E*, karaya type D; *F*, MC; *G*, CMC.

[1] An additional 50 ml. of medium was added to keep membrane submerged.

SUMMARY

1. New and improved *in vitro* tests are described for the evaluation of hydrophilic laxatives. These tests measure swelling and water retention in media reasonably similar to those of the stomach and intestinal tract. *In vivo* conditions are further simulated by a novel use of polyethylene glycol as an osmotic agent.

2. Commercial preparations of karaya and psyllium and the synthetic agents methylcellulose (MC) and sodium carboxymethylcellulose (CMC) are compared by means of these tests.

3. One significant difference between the two synthetics was observed: When agitated in simulated gastric juice, MC goes into solution slowly but CMC remains essentially insoluble.

4. The physicochemical properties of the cellulose derivatives give them two advantages over the natural gums: greater hydrophilic capacity and the formation of viscous, homogeneous solutions. The former makes a more efficient bulk laxative. The latter permits uniform distri-

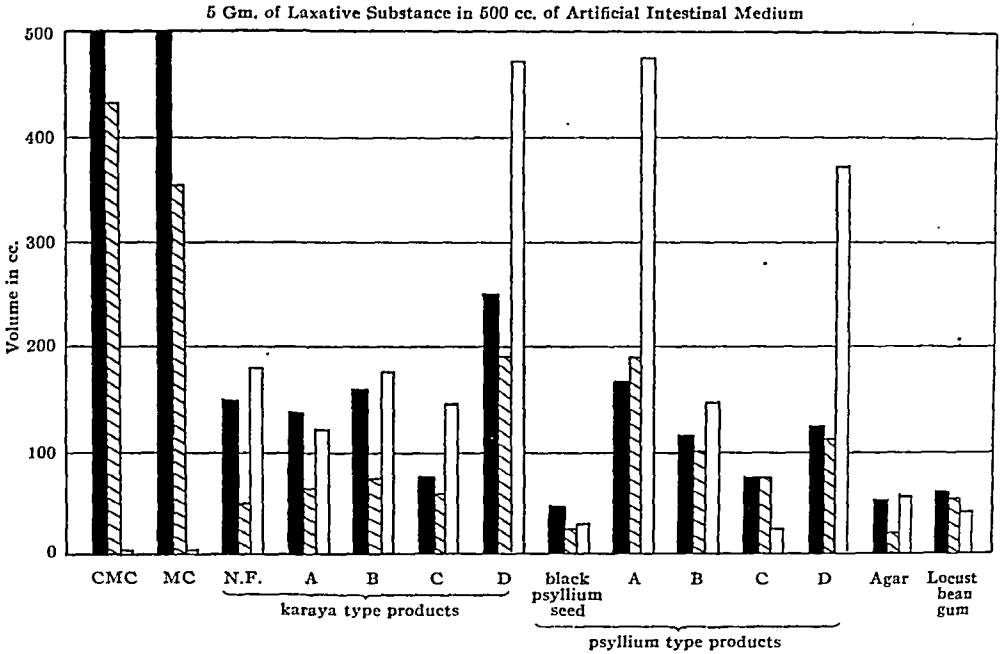

Fig. 3.—Gel volume and liquid absorption (method I). 5 Gm. of laxative substance in 500 cc. of artificial intestinal medium. Key: black, gel volume; shaded, liquid retained on glass wool; white, liquid retained on cheesecloth. Substances indicated above as karaya type products and psyllium type products A to D, inclusive, represent leading commercial products available under various trade names.

applied to dissimilar substances. Method III, devised to minimize the influence of these conditioning factors and to approximate *in vivo* conditions, gives results which seem more significant. As shown in Fig. 5, the cellulose derivatives are clearly superior in hydrophilic properties; the relative efficacy of the various karaya and psyllium preparations differs widely with the source and brand of the product tested.

NEW "HYDROPHILIC" TEST

The factors which limit the value of Methods I and II are viscosity and nature of the gelatinous mixtures formed, differing solubilities in various media, type of filter used, and degree of agitation. A search was therefore made for a way to minimize these differences. Ivy and Isaacs (3) were probably attempting this when they compared water-holding properties by measuring resistance to drying over sodium hydroxide in a vacuum desiccator. It seemed to us, however, that resistance to loss of water through a membrane under the influence of a hypertonic solution might not only solve these problems but also give a reasonable indication of the ability of the gel to retain water against osmotic pull of the body fluids within the intestinal wall.

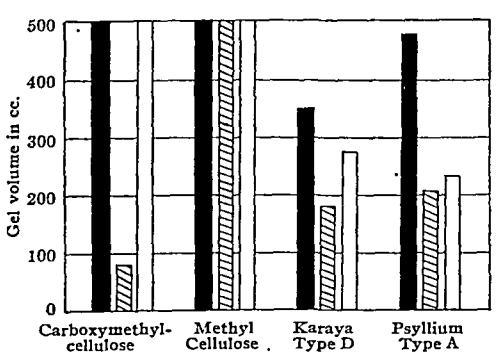

Fig. 4.—Gel volume with increased agitation (method II). Key: black, water; shaded, artificial gastric juice; white, artificial intestinal juice.

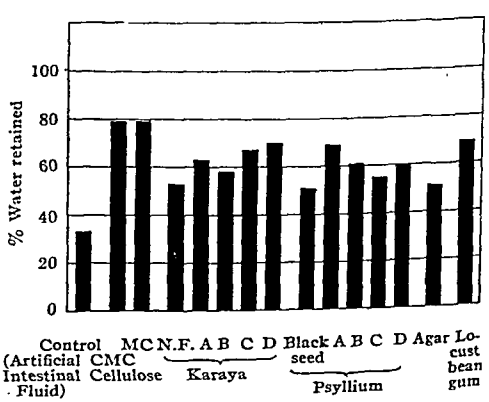

Fig. 5.—Water-holding capacity—against osmotic pull (method III). Forty-eight hour reading.

TABLE I.—GEL VOLUME AND LIQUID ABSORPTION

	Water			Artificial Gastric Juice		Artificial Intestinal Juice		
	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Consistency of Material on Filter	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Liquid Retained on Cheese Cloth, ml.
I. Cellulose Type								
CMC	500	470	Viscous sol	[a]	40	500	430	0
MC	[b, c]	[c]	[c]	[b, c]	[c]	500	355	0
II. Karaya Type								
Karaya, N. F.	205	80	Stiff and lumpy	95	35	150	50	180
A	195	90	Stiff, granular gel	95	40	135	65	120
B	270	140	Stiff, granular gel	115	80	160	75	175
C	180	120	Stiff, granular gel	60	40	75	60	145
D	350	230	Viscous, granular gel	165	125	250	190	470[d]
III. Psyllium Type								
Psyllium Seed Black	55	25	Stiff gel	30	10	45	25	30
A	370	310	Slightly viscous	180	175	165	190	475[d]
B	125	110	Slightly viscous	100	95	115	100	145
C	90	65	Slightly viscous	80		75	75	25
D	175	165	Watery	105	90	125	110	370[d]
IV. Agar	60	45	Soft gel	40	20	50	20	55
V. Locust Bean Gum	133	155	Watery	75	80	60	55	40

[a] Carboxymethylcellulose is converted to the free acid, which is insoluble.
[b] Material floats on surface of liquid in a partially swollen condition.
[c] Does not go completely into solution.
[d] Gum forms film on cheesecloth that prevents unbound liquid from draining.

Method III was, therefore, devised. As shown in Fig. 1, the gel or sol was separated by a cellophane membrane from a hypertonic solution, and the loss of liquid from the gel or sol was measured by the overflow from the osmometer. The remarkably superior water-holding capacity of the synthetic cellulose derivatives is evident from the data in Fig. 5. For comparative purposes, observations were made at forty-eight-hour intervals since, as shown in Fig. 6, the more effective substances approach an equilibrium at that time. Thus longer periods of observation (e.g., one-hundred-twenty hours) were unnecessary.

The use of a polyethylene glycol as a hypertonic agent deserves some comment. This substance was chosen because its large molecular size minimizes its passage through the cellophane membrane. Tests with the 30% solution indicated that after seventy-two hours not more than 1.1% of the polyethylene glycol has passed through the membrane into distilled water on the other side. This concentration makes significant readings obtainable within a relatively short time. The tonicity of 30% solution was calculated as 5.3 on the basis of a freezing-point depression of 2.96° C.

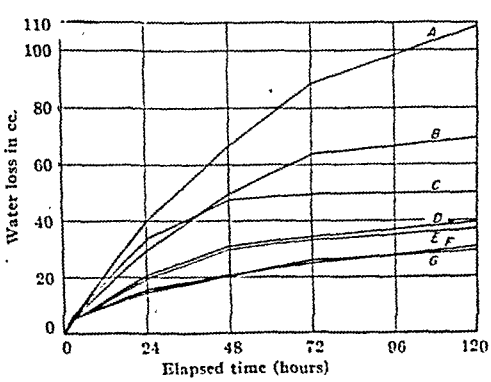

Fig. 6.—Water-holding capacity—against osmotic pull. Water loss rate. Key to curves: *A*, artificial intestinal juice[1] (control); *B*, psyllium seed black; *C*, karaya; *D*, psyllium type A; *E*, karaya type D; *F*, MC; *G*, CMC.

[1] An additional 50 ml. of medium was added to keep membrane submerged.

SUMMARY

1. New and improved *in vitro* tests are described for the evaluation of hydrophilic laxatives. These tests measure swelling and water retention in media reasonably similar to those of the stomach and intestinal tract. *In vivo* conditions are further simulated by a novel use of polyethylene glycol as an osmotic agent.

2. Commercial preparations of karaya and psyllium and the synthetic agents methylcellulose (MC) and sodium carboxymethylcellulose (CMC) are compared by means of these tests.

3. One significant difference between the two synthetics was observed: When agitated in simulated gastric juice, MC goes into solution slowly but CMC remains essentially insoluble.

4. The physicochemical properties of the cellulose derivatives give them two advantages over the natural gums: greater hydrophilic capacity and the formation of viscous, homogeneous solutions. The former makes a more efficient bulk laxative. The latter permits uniform distri-

bution through the intestinal contents and should eliminate any tendency to produce intestinal blockage.

5. Because of the general superiority of the synthetic substances over the natural products and specifically because CMC is insoluble in simulated gastric juice, it is concluded that for use as a bulk laxative CMC is the most promising substance of all those tested.

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Effect of the Rate of Injection of Digitalis Upon Its Lethal Dose in Guinea Pigs*,†

By TOM S. MIYA and HARALD G. O. HOLCK‡

Eighty-four male guinea pigs were divided into seven groups of twelve each and anesthetized with urethane. Each group was tested with varying concentrations of U. S. P. Reference Standard Digitalis (1942) Tincture, using normal saline solution as the diluent. The effects of varying the concentration of digitalis tincture are reported. The results obtained are compared with those of other investigators and recorded in this report.

ALTHOUGH the specifications for the cat in the assay of digitalis have been relaxed by allowing a wider weight range in the latest revision of the U. S. P. (1) it has become increasingly difficult to obtain cats falling within these limits. Therefore, interest has recently been revived in the possible use of the guinea-pig method introduced by Knaffl-Lenz (2) as a substitute. Several recent studies have been made dealing with the use of this animal (3, 4) to which we shall refer in the discussion.

The purpose of this study was to ascertain the effects of varying the concentration of the digitalis tincture and to compare the results with those of other investigators and also with those obtained in the cat studies of Holck, Kimura, and Bartels (5).

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Presented to the Scientific Section of the A. PH. A. San Francisco meeting, Aug. 1948.
† This project was aided by a grant to the Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, from Ciba Pharmaceutical Products, Inc.
‡ We are indebted to Professor E. Fullerton Cook for supplies of U. S. P. Reference Standard Digitalis Powder (1942) and to Professor C. T. Blunn for aid in the mathematical analysis.

EXPERIMENTAL

Eighty-four male guinea pigs were used in our main study on the effect of varying concentrations and fourteen in the second study on the effect of alcohol on the lethal dose. All the animals were obtained from the same source (Scottdale, Pennsylvania) so as to prevent as much as possible any variations in lethal dose which might occur due to difference in strain or sex, as well as any extraneous and unknown causes as reported in the use of cats by Bliss and Hanson (6). Seven different dilutions of the digitalis tincture, prepared according to the U. S. P. XIII and diluted with physiological saline solution were injected. From the lowest concentration, these increased in strength by 41.42%, giving an increase of 100% for each alternate concentration. To avoid complications which might occur if the animals were tested over a long period of time, seven animals forming a group were usually injected within a period of one week and, in no case, more than two weeks, with a concentration of 1.60, 2.26, 3.20, 4.53, 6.40, 9.05, and 12.80% of the tincture of digitalis, respectively. The order of testing the dilutions was varied with the aid of 7 x 7 Latin squares as given in the tables of Fisher and Yates (7). Because there have been conflicting reports of the effect of the weight of the animals on the lethal dose (2, 3, 8) it was decided to use a narrow range of weights (480–600 Gm.), and to distribute the animals in such a manner that the average weight in each group was nearly the same. A 25% solution of urethane was used to produce anesthesia. An initial dose of 1.0 Gm./Kg., was administered intraperitoneally and supplemented with small doses as needed. All the tests were carried out between October 17, 1947, and January 16, 1948, a period of three months.

The general procedure was that of the U. S. P. XIII (1) except that injections were made into the jugular instead of the femoral vein. Although the Pharmacopœial fasting range for cats is sixteen to

twenty-eight hours, our variations were kept within twenty to twenty-eight hours. The tests were conducted with tinctures not older than seventeen days, whereas the Pharmacopœial tolerance is thirty days. Determination of the end point was difficult because irregular and labored respiration set in before cessation of the heart beat and because the beats became sporadic before their final stoppage. The injection which caused the final stoppage of the heart was determined by auscultation and a subsequent inspection of the heart. All animals were examined and results with any guinea pigs showing gross pathological changes of the lungs were disregarded. This was done because of the finding of Holck, Smith, and Schuler (9) that cats with lung abnormalities died with a lower dose of digitalis.

RESULTS AND DISCUSSION

The main experimental data are summarized in Tables I and II.

Hildebrandt (11) and Maresh and Farah (12) found that with Strophanthin-G and various digitalis glucosides, the lethal dose was high in cats with high rates of injection and low with slow rates of injection using diallylbarbituric acid (Dial) for anesthesia, which is in general the reverse of the results obtained by Østling (10) and in this study upon guinea pigs using urethane anesthesia.

In comparing the reports of lethal doses of digitalis on guinea pigs by other workers we found that in no case did the doses agree satisfactorily with our results. Allmark and Bachinski (3) reported the following: 132.5, 125.0, 139.0, 127.9, and 130.0 mg./Kg. of digitalis per guinea pig with concentrations 2.50, 3.33, 5.00, 6.67, and 10.00%, respectively, using urethane anesthesia. There was no significant difference in mean lethal doses with the five concentrations. In the comprehensive investigation of Braun and Siegfried (13) it was noted that the lethal dose was high for light, and low for heavy animals. Much greater uniformity of response was obtained after applying a size factor. Because the body

TABLE I.—SUMMARY OF RESULTS FROM TESTS WITH U. S. P. REFERENCE STANDARD DIGITALIS (1942) IN 84 GUINEA PIGS UNDER URETHANE ANESTHESIA[a]

Concentration of Tincture, %	Mean Weight, Gm.	Mean No. of Injections	Mean Lethal Dose, Mg./Kg. Arithmetic[b]	Mean Lethal Dose, Mg./Kg. Geometric[c]
A. 1.60	513	113	180.7 ± 8.2 (4.5%)	178.7 ± 7.8 (4.4%)
B. 2.26	524	77	174.9 ± 8.7 (4.9%)	172.6 ± 8.5 (4.9%)
C. 3.20	527	54	173.8 ± 6.6 (3.8%)	172.7 ± 6.5 (3.8%)
D. 4.53	520	38	171.0 ± 6.6 (3.9%)	169.6 ± 6.7 (3.9%)
E. 6.40	525	25	157.9 ± 7.7 (4.9%)	155.9 ± 7.5 (4.9%)
F. 9.05	521	16	144.8 ± 6.7 (4.6%)	143.2 ± 6.2 (4.3%)
G. 12.80	520	11	146.1 ± 6.4 (4.3%)	144 6 ± 6.3 (4.3%)

[a] Twelve guinea pigs constitute each subgroup.
[b] The standard error in terms of percentage of the average is given in parentheses.
[c] The standard error for the geometric mean was calculated from the formula $2.3026\ (LD_{50})S_y$. S_y is the standard error of the mean log-dose.

The average lethal dose increased in general as the concentration of the tincture decreased with the exception of the highest concentration (12.80%) which gave a slightly higher lethal dose than the next lowest concentrations.

In comparing the results with those of the cat studies of Holck, *et al.* (5), we have omitted the highest concentration used in our study in the final analysis of comparison as this concentration was not employed in the cat studies.

A curved line with a negative slope (Fig. 1) was found to best fit the plotted points (log concentrations against geometric log doses) as contrasted to a straight line with a positive slope obtained by Holck, *et al.* (5), in cats with the identical first five concentrations also using urethane anesthesia. A curve similar to ours with guinea pigs, likewise under urethane anesthesia, was obtained by Østling (10) who observed that the lethal dose of Strophanthin-G in guinea pigs rose sharply up to 150 minutes of injection time and then became constant. In our case the leveling of the curve occurred between 25 and 38 injections (125 and 190 minutes, respectively). The primary comparisons are concerned with studies in which urethane anesthesia was employed, because it has been repeatedly shown that the choice of anesthetic makes a difference in the fatal dose of digitalis (5, 9).

TABLE II.—MEAN LOG-DOSES (GEOMETRIC) FOR DATA GIVEN IN TABLE I AND PLOTTED IN FIGURE 1

Log-Concentration Digitalis	Mean Log-Doses per Guinea Pig in Mg./Kg. for the Six Lowest Concentrations
0.204	0.25223
0.355	0.23711
0.505	0.23726
0.656	0.22933
0.806	0.19276
0.957	0.15609

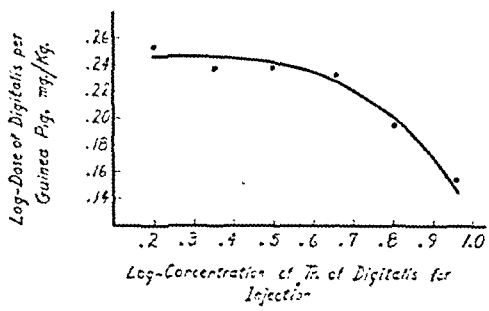

Fig. 1.—A plot of the mean log-doses for data presented in Table II.

weights in our study were well distributed within a narrow range we have used the conventional method of calculating the lethal dose. It is of some interest to note that with each of three digitalis powders studied by Braun and Siegfried the lethal dose of digitalis was lower with urethane than with ether anesthesia, whereas in the cat it has been repeatedly reported that the fatal dose of digitalis with urethane anesthesia was significantly higher than with ether anesthesia (5, 9, 14). These authors also reported that source, sex, and fasting had no appreciable effect upon digitalis toxicity in the guinea pig. It is also worthy of note that the three collaborators under the supervision of the Committee of Physiological Testing of the Scientific Section of the A. PH. A., using controlled conditions and employing local anesthesia at the site of injection, reported varying lethal doses of 125.1, 182.2, and 135.5 mg./Kg. of digitalis, respectively, with a concentration of 1:15 (4).

The mean lethal doses for the first 6 and second 6 animals used in each group were calculated. The average doses increased for groups A, B, C, and G for the second 6 animals and decreased for D, E, and F, thus no seasonal variation was evident during the testing period.

Determination of whether the higher alcoholic content of the stronger concentrations was responsible for the low mean lethal doses obtained in these groups (E, F, G) was made by testing fourteen animals (seven in each group) in a subsequent study with a concentration of 4.53%, to which enough alcohol was added to make the concentration of alcohol equal that of the 12.80% tincture. This addition of alcohol did not cause any significant added toxicity (148.8 mg./Kg. for the regular dilution and 144.8 mg./Kg. for the tincture with added alcohol). However, in this second test the fatal doses were significantly lower than those obtained in the main investigations (171.0 mg./Kg.), Table I.

The difficulty in obtaining a sharp end point and the unexplained variations in fatal dosage in this investigation and the lack of agreement of our results with those of other workers and among other investigators themselves would seem to make the guinea pig less suitable than the cat for the assay of digitalis.

SUMMARY

1. Eighty-four male guinea pigs were anesthetized with 1.0 to 1.2 grams of urethane per kilogram of body weight and subsequently injected with varying concentrations of U. S. P. Reference Standard Digitalis (1942) Tincture. The animals were arranged in seven groups of twelve each and properly randomized. They were tested with the following seven concentrations: 1.60, 2.26, 3.20, 4.53, 6.40, 9.05, and 12.80 per cent of the tincture, respectively. The dilutions were made with normal saline solution. The lethal dose of drug per kilogram of body weight was otherwise determined by the U. S. P. XIII method for assaying digitalis on cats, except that cannulation of the jugular was made instead of the femoral vein. Results from animals showing gross abnormalities of the lungs were disregarded

2. The end point was difficult to determine because the respiration became irregular considerably before the heart beats became sporadic and finally ceased.

3. No seasonal variation was demonstrable during the testing period.

4. The rate of cumulation of digitalis became more rapid with higher concentrations, hence a curved line with a negative slope seemed to fit the data best as contrasted to the straight line with the slope in the reverse direction obtained by Holck, *et al.*, in the cat for the five highest concentrations. In the present study the curve became level between 25 and 38 injections (125 and 190 minutes) indicating that lower concentrations prevent early cumulation of digitalis.

5. To ascertain whether the high alcoholic content of the stronger concentrations of the tincture were in part responsible for the lower lethal doses, a comparison between the U. S. P. Reference Standard Digitalis Tincture (4.53 per cent) and the same tincture with added alcohol was made. No significant difference in toxicity was produced by such increased alcoholic content. However, for unexplained reasons the mean lethal dose obtained for the control group in the alcohol comparison (148.8 mg./Kg.) was significantly lower than the mean lethal dose obtained in the main study with the same concentration (171.0 mg./Kg.).

6. Concentration-lethal dose correlation was poor when compared with the results of Allmark and Bachinski (3) who also used urethane anesthesia and with results of other investigators using different anesthetics.

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The Efficacy of Cetyl Trimethyl Ammonium Bromide in a Series of Ointment Bases*

By LEONARD P. PRUSAK† and ALBERT M. MATTOCKS‡

The efficacy of Cetyl Trimethyl Ammonium Bromide in a series of ointment bases has been tested and the results obtained are reported. Of the bases tested, those which were found to be most satisfactory are pectin paste and a base prepared by mixing a polyethylene glycol ("Carbowax") with water.

MUCH interest has been focused in recent years on a new group of synthetic agents collectively known as quaternary ammonium salts. They reportedly possess powerful germicidal effectiveness and are widely used at present in medicine and industry as germicides and preservatives.

Although much evidence has accumulated which demonstrates the powerful antibacterial action of quaternary ammonium salts in the form of tinctures and aqueous solutions, there is little knowledge regarding their activities in ointment form. Recently Hart and Huyck (1) evaluated Hyamine-X in five different ointment bases, using the wet and dry filter paper methods and the agar cup-plate method of assay (2). These workers concluded that Hydrophilic Ointment modified by removal of the cationic agent was the most suitable of the bases tested.

The purpose of this study was to investigate the bactericidal powers of quaternary ammonium salts in a representative series of ointment bases and to learn whether the ointment bases released the medicament in active form. For initial studies a single quaternary ammonium salt, Cetyl Trimethyl Ammonium Bromide (Baker), CTAB, was selected as a typical compound.

Previous investigations have shown that bacteriological evaluations of quaternary ammonium salts give abnormally high phenol coefficients by the FDA dilution method (3) and inconsistent results by the agar cup-plate method (4). These methods, therefore, were not considered suitable for the testing of CTAB ointments.

The quaternary ammonium salts are such powerful bacteriostatic agents that a loopful of broth used for subcultures may contain sufficient medicament to cause bacteriostasis and thus give erroneous results. To overcome this difficulty, several methods have been suggested, such as that of Klarman and Wright (5) which utilizes high dilution technique; that of Lawrence (6) which uses Suramin as an inactivating agent; and that of Quisno, Gibby, and Foter (7) which uses lecithin as an inactivator. The latter workers found that a broth containing lecithin in predetermined amounts dispersed with one-half per cent Tween-80 could be used as a subculture medium which would prevent further action of quaternary ammonium salt on surviving bacteria. This medium was named Letheen Broth.

In this study the CTAB ointments were evaluated by dispersing them in broth inoculated with *Staphylococcus aureus*, incubating the mixtures at 37° for periods of three and twenty-four hours, and then subculturing a loopful of each into Letheen Broth.

An unsuccessful attempt was made to determine the amounts of CTAB released into broth by the various ointments using the colorimetric assay of Auerbach (8, 9).

A bacteriological experiment was conducted to show the effect of blank ointment bases upon effective concentrations of CTAB.

EXPERIMENTAL

Five ointment bases official in the United States Pharmacopœia XIII and the National Formulary VIII and one unofficial base were used in this investigation. Hydrophilic Ointment U. S. P., a typical oil-in-water emulsion base, Hydrophilic Petrolatum U. S. P., a water-in-oil emulsion base, Simple Ointment U. S. P., a petrolatum-type base, Tragacanth Glycerite N. F. and Pectin Paste N. F., mucilaginous bases, and Carbowax Ointment Base (prepared by mixing Carbowax 1500 with 10% water), a polyethylene glycol base, were selected as a representative group of ointment bases.

The ointments were prepared by incorporating 1.0 Gm. CTAB into a sufficient quantity of base to make 100 Gm. ointment, using gentle heat or a small amount of water when desirable.

* Received July 17, 1948, from the School of Pharmacy, Western Reserve University, Cleveland, Ohio.
This paper [illegible] faculty of the Gradu[illegible] ty by Leonard P. F[illegible] ments for the Degre[illegible]
† Fellow, [illegible] Education, 1947–1948.
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Bacteriological Assay.—The method adopted for this investigation was one involving the inactivation of quaternary ammonium salts by lecithin.

In the bacteriological tests a 0.5 Gm. sample of ointment containing 1% CTAB was to be used in a total mixture of 6 Gm. It was calculated that one loopful (0.05 ml.) of this mixture would contain at most 4.1 x 10^{-5} Gm. of CTAB. When transferred to the subculture medium, the maximum concentration of CTAB would become 1:120,000. In order to prove that the Letheen Broth used in the subcultures was sufficient to inactivate the maximum amount of quaternary salt which might be present, the following experiment was performed: One ml. of 1:11,000 CTAB was added to each of six tubes containing 5 ml. Letheen Broth (0.07% vegetable lecithin) and 0.5 ml. of a 24-hour culture of *S. aureus* (10), resulting in a final CTAB concentration of about 1:71,000. The mixture was incubated twenty-four hours at 37° and read for turbidity. A parallel series was run using nutrient broth that did not contain lecithin. All tubes containing lecithin showed bacterial growth while all those without lecithin were growth-free. This demonstrated that the Letheen Broth effectively neutralized the maximum amounts of CTAB that might be present in subcultures.

The technique employed for antibacterial testing of ointments was as follows:

Tubes containing 5 ml. quantities of sterile nutrient broth were inoculated with 0.5 ml. of a twenty-two- to twenty-six-hour culture of *S. aureus*. One-half gram of 1% CTAB ointment, accurately weighed on high gloss paper, was added to the inoculated broth and the mixture incubated for three-hour and twenty-four-hour periods at 37°. At the end of each incubation, 4-mm. loopfuls were subcultured into 5-ml. Letheen Broth, shaken thoroughly with a hand-operated shaking machine and incubated for forty-eight hours. The tubes were then read for turbidity. Results are shown in Table I.

TABLE I.—GERMICIDAL EFFECTIVENESS OF 1% CTAB OINTMENTS[a, b]

Ointment (Containing 1% CTAB)	Bacterial Growth: 3-Hr. Incubation		24-Hr. Incubation	
Hydrophilic Ointment	+++	+++	+++	+++
Hydrophilic Petrolatum	+++	+++	+++	+++
Simple Ointment	+++	−−−	+−−	+++
	−−+		−−−	−−−
			−−−	−−−
			−−+	
Tragacanth Glycerite	−−−	−−+	−++	−−+
	−−−	+++		
Pectin Paste	−−−	−−−	−−−	−−−
Carbowax Ointment	−−−	−−−	−−−	−−−
	−−−	−−−		

[a] + indicates turbidity (growth); − indicates no turbidity.
[b] Each symbol represents one test.

Interference Tests.—To obtain a more accurate picture of the germicidal activity of CTAB in ointment bases, it became necessary to determine the extent of interference, if any, imparted by the various ointment bases. To accomplish this, bacteriological tests were carried out in the following manner:

Analytically accurate dilutions of CTAB ranging in concentration from 1:5000 to 1:140,000 were prepared and tested for bactericidal activity. A 1-ml quantity of each dilution was added to a tube containing 5 ml. nutrient broth and 0.5 ml. of a twenty-four-hour culture of *S. aureus*, and the mixture was incubated for twenty-four hours. At the end of this time 0.05 ml. of the mixture was subcultured in 5 ml Letheen Broth, and this was incubated for forty-eight hours. A second series of tubes containing the same amounts of CTAB was run under the same conditions except that 0.5 Gm. of the blank ointment base was added after the CTAB had been thoroughly mixed with the broth and before the mixture was inoculated with bacteria. Corresponding results were obtained by a duplication of the experiment. Results are shown in Table II.

Attempted Assay of Released Germicide by Colorimetric Procedure.—Ointments were added to broths in the usual way, and, after suitable incubation, were assayed by the colorimetric method of Auerbach. Results were inconsistent with bacteriological findings, and further investigation demonstrated that the ointment bases adsorbed in varying degrees the CTAB-bromphenol colored complex. This explained the erratic colorimetric results and proved that the Auerbach method is not suitable for ointments without special modifications to overcome adsorption effects.

DISCUSSION

The experimental work was planned to show: (1) the relative efficacies of six ointments containing 1% CTAB, (2) whether decreased effectiveness of the germicide contained in the ointments was due to failure of the base to release the medicament. In order for an ointment to be considered efficient by this method of evaluation, it must be capable of releasing the germicidal agent into nutrient broth. Also, the ointment base itself must not interfere with or inhibit the action of already-released CTAB.

The bactericidal tests (Table I) showed clearly the relative efficacies of the six ointments tested by demonstrating the bactericidal action after three and twenty-four hours' contact with the bacteria. These results represent a summation of the various physical and chemical properties of the ointment bases that affect the activity of CTAB on bacteria.

The interference measurements (Table II) showed the maximum dilution of CTAB free of ointment base that had bactericidal activity as compared with the same dilutions when mixed with ointment bases. This, in effect, was a direct measurement of the ability of the base to remove CTAB from solution, by adsorption or precipitation, or to prevent, in some way, the action of CTAB on bacteria.

Hydrophilic Ointment U. S. P. showed no bactericidal activity in spite of prolonged contact with bacteria. The interference measurements showed that this ointment base prevented the antibacterial action of even the most concentrated solution of CTAB tested. Since this ointment is an oil-in-water emulsion containing the germicidal compound in the aqueous phase, it may be assumed that the CTAB molecules should have ample opportunity to come in

TABLE II.—INTERFERENCE TESTS[a, b]

Conc. CTAB Added	Without Ointment Base	With Ointment Bases Added: Hydrophilic Ointment	Hydrophilic Petrolatum	Simple Ointment	Tragacanth Glycerite	Pectin Paste	Carbowax Ointment
1:5000	—	M	M	S	—	—	—
1:10,000	—	M	M	S	S	—	—
1:20,000	—	H	H	S	S	—	S
1:40,000	S	H	H	M	M	S	S
1:60,000	S	H	H	M	H	S	M
1:80,000	M	H	H	M	H	M	M
1:100,000	M	H	H	H	H	M	H
1:120,000	H	H	H	H	H	H	H
1:140,000	H	H	H	H	H	H	H

[a] Each symbol represents a duplicate set of tests.
[b] S = Slight growth; M = Moderate growth; H = Heavy growth; — = No growth.

contact with the bacteria, other factors not being considered. However, it is known that sodium lauryl sulfate (which is present in Hydrophilic Ointment) forms a precipitate with CTAB in aqueous solution, and it is possible that this phenomenon is responsible for the lack of activity of CTAB in this base. This view is substantiated by the results of Hart and Huyck (1) which showed that Hydrophilic Ointment without sodium lauryl sulfate released effective amounts of a quaternary ammonium germicide.

Hydrophilic Petrolatum U. S. P. also gave negative bactericidal results, and prevented CTAB activity in the interference tests. There is no indication that a specific chemical reaction takes place between CTAB and any of the ingredients of the ointment, such as occurs with sodium lauryl sulfate in Hydrophilic Ointment. However, this base has a powerful inactivating or adsorption effect, as shown by interference tests, and may not be considered suitable as a vehicle for CTAB.

Simple Ointment U. S. P. showed bactericidal activity in about 50% of the three-hour tests and in about 80% of the twenty-four hour tests. This makes it appear that the amount of CTAB released from the ointment gives a concentration of germicide very near the minimum effective range. Interference measurements substantiate the bactericidal tests, since the ointment base inactivated CTAB to such an extent that slight bacterial growth was noted with the three highest concentrations and moderate to heavy growth with lower concentrations of germicide. These results indicate that Simple Ointment has an adsorptive or inactivating power for CTAB which prevents release of the latter in effective amounts.

Tragacanth Glycerite N. F. proved to be of doubtful effectiveness by bactericidal evaluations. Interference measurements indicated that the base prevents the action of CTAB in concentration of 1:60,000 or less. If all of the germicide contained in the ointment had been released into the broth, the resultant concentration would have been 1:1200. It is apparent, therefore, that most of the CTAB remained adsorbed on the ointment base, leaving in solution a noneffective concentration.

Pectin Paste N. F. proved to be an effective carrier of CTAB. It had bactericidal action on both three-hour and twenty-four hour tests. Interference measurements proved that this ointment interfered in no way with action of the germicide, a solution containing ointment being equally as effective as an aqueous solution of germicide alone. This makes it appear that Pectin Paste is an ideal base for CTAB ointment.

Carbowax Ointment had bactericidal properties in both three and twenty-four hour tests. Interference measurements indicated that Carbowax does prevent the action of or adsorb CTAB to a very limited extent. For all practical purposes, it is believed that Carbowax is as valuable a carrier of CTAB as Pectin Paste.

CONCLUSIONS

Hydrophilic Ointment U. S. P. and Hydrophilic Petrolatum U. S. P. are not satisfactory carriers of CTAB. Simple Ointment U. S. P. and Tragacanth Glycerite N. F. release such small amounts of quaternary ammonium salt from their combinations that they cannot be considered useful bases for CTAB.

Pectin Paste N. F. and Carbowax Ointment readily release CTAB in an active form, demonstrating that they are suitable bases for quaternary ammonium salts.

The method of bactericidal evaluation of quaternary ammonium compounds devised by Quisno, Gibby, and Foter can be conveniently and satisfactorily applied to ointments.

The Auerbach Method of analysis of quaternary ammonium salts is not satisfactory for the assay of ointments.

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The Synthesis of Pyridine Derivatives Possessing Possible Emetic and Rodenticidal Activity*

By WILLIAM D. JONES,† GLENN L. JENKINS, and JOHN E. CHRISTIAN

Since the methoxy group is present in emetine, in a patented emetic, and in several nauseant benzene derivatives it is possible that this group may be responsible for the emetic action of these compounds. In this report the syntheses of several methoxy derivatives of pyridine are described.

A RODENTICIDE which also possessed emetic properties would be considerably more advantageous than the usual rat poison which is equally fatal to higher animals. Its advantage would be in the fact that rats cannot regurgitate while other animals can. Hence, if the emetic-poison were taken by household pets, such as dogs or cats, the poison would promptly be regurgitated, but to rats, which do not have this power, it would be fatal.

Since the methoxy group is present in emetine (an alkaloid which can cause emesis by irritating the stomach mucosa), in a patented emetic consisting of 6,7-dimethoxyisoquinoline (1) and in several nauseant methoxy benzene derivatives prepared by some German investigators seeking antimalarials (2), it would appear that the methoxy group is responsible for the emetic activity exhibited by these compounds.

On the basis of this information, it was decided to investigate the influence of the methoxy group on the pyridine nucleus. It was hoped that the addition of the methoxy group to pyridine would lend emetic properties and with such toxic groups as chlorine, produce a product which might be a useful rodenticide.

EXPERIMENTAL

1. 5-Chloro-2-aminopyridine and 3,5-dichloro-2-aminopyridine.—The procedure used was that of Chichibabin and Egorov (3).

2. The attempted synthesis of 5-methoxy-2-aminopyridine.—A series of experiments was performed in an attempt to prepare 5-methoxy-2-aminopyridine from 5-chloro-2-aminopyridine by modification of the procedure of Schickh, Binz, and Schulz (4). The following is representative of these experiments which yielded about 45–50% of a colorless oil. The other experiments (using lower temperatures and pressures and altering tl reactions) yielded a small quantity of this oil ar large amounts of starting materials. One hundre grams of 5-chloro-2-aminopyridine, 130 Gm. sol sodium methoxide (95%), 500 ml. methanol, ar 25 Gm. copper sulfate were charged to the ste liner of a pressure bomb. The liner was placed i the bomb and heated under an atmosphere of nitr gen at 300° for three hours. After cooling, tl contents were removed from the liner, filtered, tl solvent removed under vacuum, the residue treate with a little water and extracted with ether. Tl ether was removed under vacuum and the residu distilled using vacuum. The product was a colo less oil boiling at 203–204° (at atmospheric pre sure).

Anal.: Calcd. for $C_6H_8N_2O$: N, 22.6%; C 58.0%; H, 6.45%. Found: N, 25.0%; C 66.0%; H, 7.48%.

The analysis clearly indicated that the compoun sought was not obtained. When the analysi boiling point, and the melting point of the picrat of this compound and 2-methylaminopyridine wer compared, it was concluded that the compound pre pared by this procedure was 2-methylaminopyr dine.

	Unknown	2-Methylaminopyridin
Carbon	66.0%	66.7%
Hydrogen	7.48%	7.42%
Nitrogen	25.0%	25.9%
Boiling point	203–204°	200–201° (10)
Melting point of picrate	192°	190° (10)

3. 5-Chloro-3-nitro-2-aminopyridine.—Th procedure followed was that of Chichibabin an Egorov (3).

4. 5-Chloro-3-nitro-2-bromopyridine.—Craig' procedure (5) for the preparation of 2-bromopyridin from 2-aminopyridine was followed. Five grams o 5-chloro-3-nitro-2-aminopyridine was dissolved i 14 ml. of cold hydrogen bromide (48%). Four an three-tenths grams of bromine was cautiousl added to the stirred reaction mixture. A solutio of 4.9 Gm. of sodium nitrite in 7 ml. of water wa added dropwise and the mixture stirred for two hours Ten and nine-tenths grams of sodium hydroxide i 25 ml. of water was added causing a precipitate t form. The precipitate was filtered off, dissolved i alcohol, decolorizing charcoal added, the charcoa removed by filtration, and the filtrate chilled. Th product, yellow tinged white plates, melted at 77-79°. The yield was 14.6% of theoretical.

Anal.: Calcd. for $C_5H_2BrClN_2O_2$: N, 11.8% Found: N, 11.8%.

5. 5-Chloro-3-nitro-2-hydroxypyridine.—The procedure used was a modification of that used by Phillips (6) for preparing 5-nitro-2-hydroxypyridine. Twelve grams of 5-chloro-3-nitro-2-aminopyridine was dissolved in 100 ml. water and 50 ml.

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sulfuric acid. This solution was chilled and 10 Gm. sodium nitrite in 20 ml. of water was added dropwise with vigorous stirring. The product precipitated out during the addition of the sodium nitrite solution. The crystals were filtered off, washed with water, and dried. The product consisting of light yellow needles weighed 10.3 Gm. (85.5% of theoretical yield) and melted at 231–234°. The melting point was not changed after several recrystallizations from benzene and alcohol.

Anal.: Calcd. for $C_5H_3ClN_2O_3$: N, 16.1%. Found: N, 15.8%.

Several attempts to reduce catalytically 5-chloro-3-nitro-2-hydroxypyridine in different solvent combinations using a Parr Catalytic Hydrogenation apparatus produced only tarry residues.

6. 5 - Chloro - 3 - nitro - 2 - methoxypyridine.—The procedure followed was a modification of the method developed by Bickel (7) for preparing methoxypyridine derivatives. Twenty-five grams of 5-chloro-3-nitro-2-hydroxypyridine was suspended in 100 ml. of benzene. To the cooled and stirred suspension, 17.5 Gm. of diazomethane in 637 ml. of benzene was added slowly. The reaction mixture was allowed to stand overnight at room temperature. The solvent was removed under vacuum and the residue was purified by sublimation (15 mm. pressure). The product was 16.8 Gm. (62.3% of theoretical) of pale yellow needles melting at 84–88°.

Anal.: Calcd. for $C_6H_5ClN_2O_3$: N, 14.9%. Found: N, 14.6%.

When 5-chloro-3-nitro-2-methoxypyridine was reduced catalytically (in a Parr Catalytic Hydrogenation apparatus), white needles melting at 168–170° were obtained after purification by sublimation.

Anal.: Calcd. for $C_6H_7ClN_2O$: N, 17.7%; C, 45–6%; H, 4.4%. Found: N, 21.8%; C, 49.5%; H, 5.0%.

The analysis indicated that the product formed was not 5-chloro-3-amino-2-methoxypyridine. The presence of a primary amine could not be shown. The product remained unidentified.

7. The attempted synthesis of 3,5-dimethoxy-2-aminopyridine.—Three attempts were made to react 3,5-dichloro-2-aminopyridine with sodium methoxide at high temperatures and pressures in a pressure bomb using copper sulfate as a catalyst, but no product was attainable.

8. The synthesis of 3,5-dibromopyridine.—The procedure followed was that of McElvain and Goese (8). Four attempts (modifications of the procedure of Koenigs, *et al.* (9), were made to react 3,5-dibromopyridine with sodium methoxide using copper sulfate as the catalyst in a pressure bomb. The reaction conditions tried were as follows: Ten hours at 200°, twenty hours at 200°, three hours at 250°, and one hour at 300°.—No product was attainable.

9. The attempted synthesis of 5-diethylamino-2-aminopyridine.—Ten grams of 5-chloro-2-aminopyridine, 17 Gm. of diethylamine, 10 Gm. copper sulfate, 5 Gm. sodium carbonate, and 500 ml. of benzene was charged to the steel liner of the pressure bomb. The liner was placed in the bomb, nitrogen added, and the bomb heated at 250° for seven hours. After cooling, the contents of the liner were removed, filtered, the solvent removed under vacuum, and the residual oil treated with water. The mixture was extracted with ether, the ether removed under vacuum, and the residue vacuum distilled. The product which distilled at 100–103° (16 mm. pressure) crystallized upon cooling and was recrystallized from ligroin. The material was white plates melting at 60°.

Anal.: Calcd. for $C_9H_{15}N_3$: N, 25.5%; C, 65.5%; H, 9.0%. Found: N, 29.7%; C, 64.5%; H, 6.9%.

The analysis proved that the product was not 5-diethylamino-2-amino-pyridine but indicated that the compound 2,2′-diamino 5,5′-dipyridyl was formed by the reaction.

Anal.: Calcd. for $C_{10}H_{10}N_4$: N, 30.1%; C, 64.1%; H, 5.4%. Found: N, 29.7%; C, 64.4%; H, 6.9%.

The proof that 2,2′-diamino 5,5′-dipyridyl was the product formed was shown by the preparation of the dibenzenesulfonamide, the tetranitro derivative, the dipicrate and through the synthesis of the compound by heating 5-chloro-2-aminopyridine with sodium carbonate and copper sulfate in the pressure bomb.

10. 2,2′-Dibenzenesulfonamide 5,5′-dipyridyl.—Eight-hundredths of a gram of 2,2′-diamino 5,5′-dipyridyl was heated on a steam bath with 1 ml. of benzenesulfonyl chloride. Five milliliters of 10% sodium hydroxide was added and the heating continued for several more minutes. The sodium hydroxide layer was decanted and neutralized. The crystalline product which precipitated was recrystallized from benzene. The product melted at 173–174°.

Anal.: Calcd. for $C_{22}H_{18}N_4O_4S_2$: N, 12.0%. Found: N, 12.2%.

11. Tetranitro 2,2′-diamino 5,5′-dipyridyl.—Five-tenths of a gram of the 2,2′-diamino 5,5′-dipyridyl was dissolved in 6.6 ml. of cold concentrated sulfuric acid. Five-tenths of a milliliter of concentrated nitric acid was added dropwise with stirring to the solution. After standing one-half hour at room temperature, the solution was poured into ice water. The precipitate which formed was filtered off, washed and recrystallized from 95% ethanol. The product melted at 164° (dec.) and consisted of yellow plates.

Anal.: Calcd. for $C_{10}H_6N_8O_8$: C, 32.8%; N, 30.6%; H, 1.6%. Found: C, 32.9%; N, 30.7%; H, 2.4%.

12. The dipicrate of 2,2′-diamino 5,5′-dipyridyl.—About 0.05 Gm. of 2,2′-diamono 5,5′-dipyridyl was dissolved in 5 ml. of 95% ethanol and heated on a steam bath. Saturated picric acid in ethanol was added, the solution cooled, and the crystals filtered off. The product, recrystallized from ethanol, melted at 244–246° (dec.).

Anal.: Calcd. for $C_{22}H_{18}N_{10}O_{14}$: N, 21.7%. Found: N, 21.7%.

13. 2,2′-Diamino 5,5′-dipyridyl.—In gathering additional proof that the product in Experiment 9 was 2,2′-diamino 5,5′-dipyridyl, 15 Gm. of 5-chloro-2-aminopyridine was heated with 18.0 Gm. sodium carbonate, 7.0 Gm. copper sulfate, and 150 ml. benzene at 300° for three hours in a pressure bomb. The product was shown to be identical with that in Experiment 9 by a mixed melting point. 2,2′-Diamino 5,5′-dipyridyl can also be prepared by heating 5-chloro-2-aminopyridine with sodium ethoxide and copper sulfate at 300° for three hours

The yields were about 1.5% of theoretical in both experiments.

14. 3,5-Dichloro-2-hydroxypyridine.—The procedure used was that of Chichibabin and Egorov (3).

15. 3,5 - Dichloro - 2 - methoxypyridine.—Seven grams of 3,5-dichloro-2-hydroxypyridine was suspended in 50 ml. of ether. One hundred and five milliliters of an ether solution containing about 5.4 Gm. of diazomethane was added slowly to the suspension. The reaction mixture was allowed to stand overnight. The solvent was removed under vacuum and the residue recrystallized from carbon tetrachloride. The product, white needles, weighed 5 Gm. (66.2% of theoretical) and melted at 137–140°.

Anal.: Calcd. for $C_6H_6Cl_2NO$: N, 7.9%. Found: N, 7.86%.

16. 5-Chloro-2-hydroxypyridine.—The procedure followed was that of Chichibabin and Egorov (3).

17. 5-Chloro-2-methoxypyridine.—Six grams of 5-chloro-2-hydroxypyridine was suspended in 50 ml. of benzene. One hundred and fifty-five milliliters of an ether solution containing about 5.7 Gm. of diazomethane was added slowly with stirring to the suspension. The reaction mixture was allowed to stand overnight. The solvent was removed and the residue (an oily crystalline mass) was crystallized from ligroin and benzene. The product, white needles, weighed 3 Gm. (45.2% of theoretical) and melted at 45–46°.

Anal.: Calcd. for C_6H_6ClNO: N, 9.85%. Found: N, 9.70%.

SUMMARY

1. Three chloro-methoxypyridine derivatives possessing possible emetic and rodenticidal activity have been synthesized, namely: (*a*) 5-chloro - 3 - nitro - 2 - methoxypyridine; (*b*) 3,5-dichloro - 2 - methoxypyridine; (*c*) 5 - chloro-methoxypyridine.

2. The following other new compounds ha been synthesized: (*a*) 5-chloro-3-nitro-2-brom pyridine; (*b*) 5 - chloro - 3 - nitro - 2 - hydrox pyridine; (*c*) 2,2′ - diamino 5,5′ - dipyridy (*d*) 2,2′ - dibenzenesulfonamide 5,5′ - dipyridy (*e*) tetranitro 2,2′-diamino 5,5′-dipyridy (*f*) 2,2′ - diamino 5,5′ - dipyridyl dipicrate.

3. When the three methoxy compounds we given a preliminary test for possible action on th emetic center in the medulla (using pigeons 5-chloro-2-methoxypyridine caused emesis i one-half hour, 3,5-dichloro-2-methoxypyridin was toxic but caused no emesis and the solubilit of 5-chloro-3-nitro-2-methoxypyridine prevente a complete test (small doses caused no emesis).

4. Quantities of both 5-chloro-2-methoxy pyridine and 3,5-dichloro-2-methoxypyridine ar available for further pharmacological testing.

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Solubility of Cinchonine and Cinchonidine in Alcohol-Water Mixtures*

By NAZIR A. CHOWDHRY and CHARLES F. BONILLA

The solubility of cinchonine and of cinchonidine in mixtures of alcohol and water in different proportions has been determined. The method employed and the results obtained are reported.

IN CONNECTION with further development work at this University of the U. S. Army portable quinine extraction plant (1), sponsored by the Department of Commerce and Reconstruction Finance Corporation, it was desirable to know the solubility of the cinchona alkaloids in alcohol-water mixtures at room and higher temperatures. These data would be useful in predicting the amount of alcohol necessary to extract the alkaloids from the Zeo-Karb bed, the amount remaining in solution in the alcohol recovery still product liquor, and the effect of varying the alcohol concentration and temperature on both of these quantities.

EXPERIMENTAL

The Ecuadorean bark employed in testing the process analyzed 1.0% quinine, 1.3% cinchonidine,

* Received April 21, 1948, from the Department of Chemical Engineering, Johns Hopkins University, Baltimore, Md.

and 2.0% cinchonine. In view of the smaller amount and much higher solubility in alcohol reported for quinine (2) only the solubilities of cinchonine and cinchonidine were believed to be necessary.

A literature survey revealed no data on intermediate alcohol concentrations, and discrepancies and uncertainties among the available figures for concentrated alcohol, as indicated in Table I.

TABLE I.—PREVIOUS SOLUBILITY DATA IN ALCOHOL

Alkaloid	Temp., ° C.	Alcohol Concentration	Solubility, % of Total Weight	Reference
Cinchonine	25° ±	96.6% ± by vol. (or 100%?)	2.04	(3)
Cinchonine	15° ±	96.6% ± by vol. (or 100%?)	0.826 (or 1.02?)	(4)
Cinchonine	20°	88.8% by vol. (S.G. 25° = 0.832)	0.855	(5)
Cinchonidine	25° ±	96.6% by vol. (or 100%?)	4.76	(3)
Cinchonidine	15° ±	96.6% by vol. (or 100%?)	4.76 (or 5.8?)	(4)

Uncertainties in alcohol concentration indicated in Table I are due to its not being stated definitely in the references cited, and in solubility to no statement as to whether volume or mass of the solvent is intended. Accordingly the desired solubilities of commercial samples of pure cinchonine and cinchonidine were determined.

An excess of the dried alkaloid was shaken frequently in tightly capped bottles with the desired composition of alcohol until 3- or 5-ml. portions of the liquid, pipetted out at about fifteen-minute intervals, weighed, and evaporated to dryness in a weighing bottle in air at 50°, showed a constant percentage of residue. Each experimental figure reported in Table II was obtained in at least two consecutive check determinations, indicating saturation had been reached. For the 50° tests a Carrier atmospheric drying cabinet, automatically maintained within less than 0.5° of this temperature, was employed.

The results are listed in Table II under 27.9°, 28.2°, and 50° C. If these solubilities are plotted as isotherms against the solvent composition, quite smooth curves result. However, the value for cinchonine at 28.2° in 50% alcohol appears to be low, though saturation seemed to have been attained as completely as at the other conditions.

To obtain approximate solubilities at other temperatures, the experimental values were recomputed to percentage of the weight of solvent, and the logarithm of this quantity plotted against the reciprocal of the absolute temperature. Due to the low concentration (in mole fraction) of these solutions, this plot should yield a straight line (2). A straight line was therefore drawn through each pair of points for the same solute and solvent, and the solubility at each desired temperature read off. These figures were recomputed to per cent based on the total weight and are listed also in Table II. The accuracy is probably least at the highest and lowest temperatures given, but should be fairly good since the lines for both alkaloids were nearly parallel, and what variation in slope occurred took place gradually in passing from one alcohol concentration to the next (except for cinchonine in 50% alcohol, which therefore was not extrapolated).

Comparing Table I with Table II it is seen that previous solubility values for cinchonine are higher than the new ones, whereas for cinchonidine they are lower.

TABLE II.—SOLUBILITY OF ALKALOIDS IN ALCOHOL-WATER MIXTURES

% by Volume of Alcohol in Solvent	Solubility of Cinchonine in Gm. per 100 Gm. of Solution, at ° C.										
	0	10	15	20	25	28.2	30	40	50	60	70
96.6	0.517	0.681	0.775	0.803	0.99	1.08	1.13	1.41	1.73	2.12	2.58
90	0.394	0.54	0.626	0.725	0.833	0.894	0.946	1.22	1.54	1.91	2.37
80	0.19	0.28	0.339	0.418	0.493	0.542	0.577	0.804	1.09	1.45	1.91
70	0.0885	0.135	0.165	0.20	0.239	0.271	0.286	0.404	0.56	0.754	1.02
60	0.049	0.075	0.092	0.114	0.136	0.153	0.163	0.23	0.32	0.433	0.586
50	...	...	...	...	...	0.0304	...	...	0.15	....	....
	Solubility of Cinchonidine in Gm. per 100 Gm. of Solution at ° C.										
	0	10	15	20	25	27.9	30	40	50	60	70
96.6	3.85	4.97	5.59	6.29	7.06	7.52	7.83	9.42	11.51	13.6	16.0
90	3.48	4.55	5.17	5.84	6.54	7.02	7.34	9.08	11.02	13.0	15.5
80	1.96	2.7	3.15	3.61	4.17	4.54	4.75	6.1	7.71	9.54	11.8
70	0.868	1.26	1.52	1.81	2.15	2.35	2.5	3.4	4.55	5.84	7.58
60	0.46	0.70	0.853	1.04	1.26	1.38	1.49	2.09	2.89	3.85	5.12
50	0.097	0.159	0.202	0.255	0.316	0.36	0.391	0.585	0.88	1.22	1.73

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Chemical Constitution and Analgetic Action*

By F. W. SCHUELER, E. G. GROSS, and H. HOLLAND

Recent advances in the pharmacology of morphine and its analogs suggest that at least part of the analgetic response of these agents is traceable to their action through the autonomic nervous system. Evidence derived from a consideration of the chemical constitutional relations involved in morphine and a host of new analogs support this view and indicate the presence of both sympathomimetic and parasympathomimetic moieties connected (in general) by the same amino nitrogen atom within the same molecule.

IN RECENT years it has been indicated that the definition of chemical constitutional requirements for a given type of pharmacodynamic action may be best elucidated when specific chemical constitutional aspects of a given molecule are made the focus of interpretation rather than vague references to the molecule as a whole. The successful application of this approach to the problem of sex-hormonal action (21, 22) and muscarinic action (19) has encouraged us to extend it to the elucidation of the relation between analgetic action and chemical constitution.

In spite of the common assertion that morphine owes its analgetic effect to a "sledge-hammer" central depressant action, certain facts stand in the way of a ready acceptance that this is the only mechanism involved. Some of these facts are outlined in the summary and discussion below.

1. The peak analgetic response to morphine is not simultaneous with its peak central depressant effect (18, 24).

2. Some animals show great central excitation with morphine in spite of a concomitant analgetic response. For example, the cat shows great central excitement with morphine, but requires a smaller (per kilo) dose of morphine for a given analgesic response than the dog, which is depressed by this drug (12).

3. The analgetic response to morphine is not decreased by amphetamine and other C. N. S. stimulants, but, on the contrary, is enhanced by these agents (10).

4. *In vitro* experiments indicate the doses of morphine and its analogs for the depression of brain respiration to be too large to explain their analgetic action in physiological doses upon a central depressant basis (2).

* Received June 2, 1948, from the Department of Pharmacology, College of Medicine, Univ. of Iowa, Iowa City.

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TABLE I.

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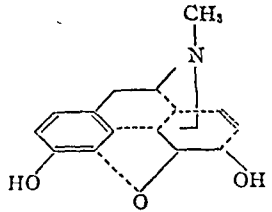
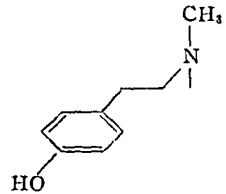
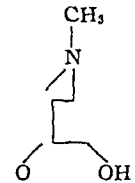
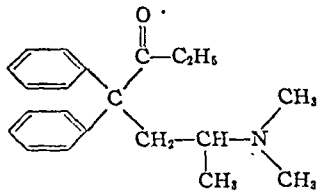
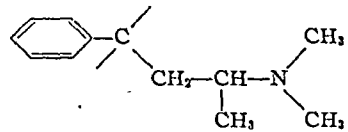
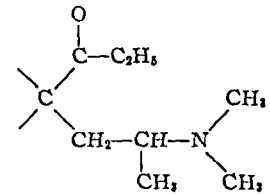
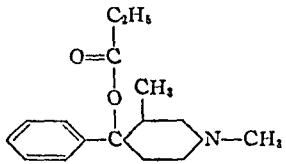
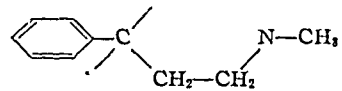
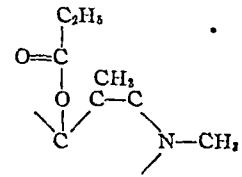
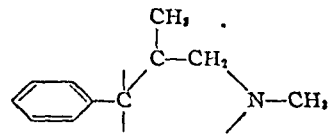
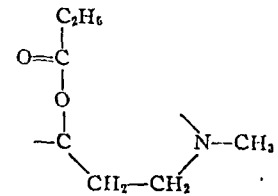
1			
2 (21)			
3			
4 (8)			

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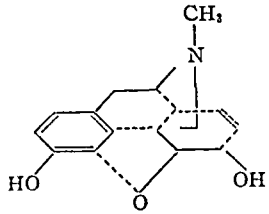
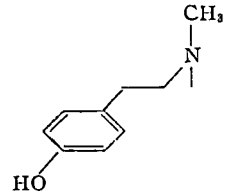
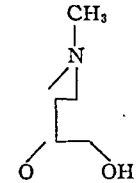
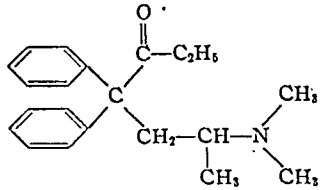
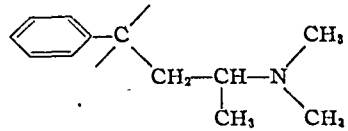
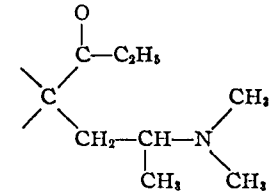
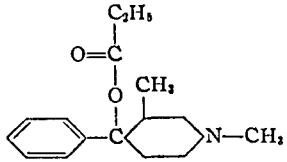
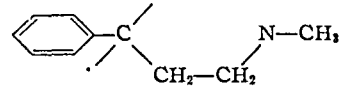
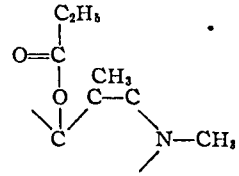
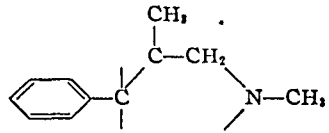
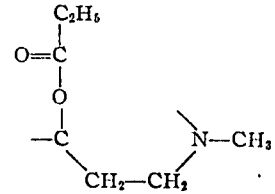
1			
2 (21)			
3			
4 (8)			

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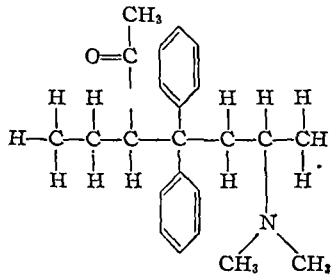
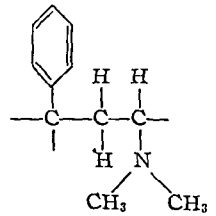
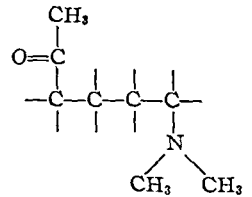
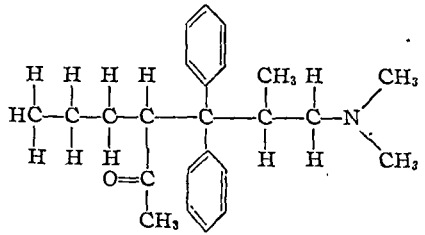
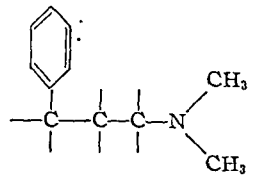
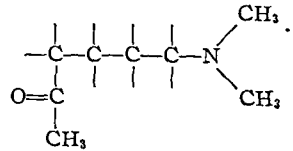
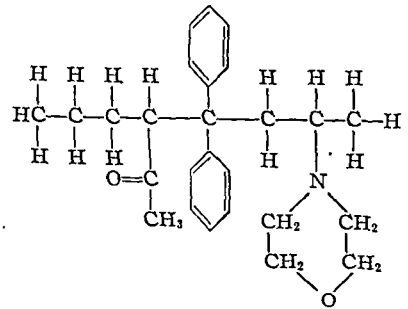
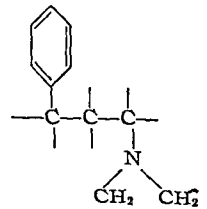
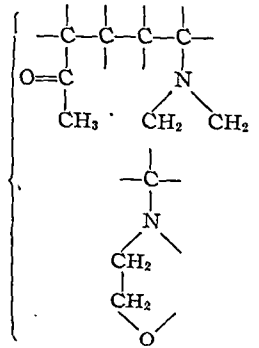
5 (23)			
6 (23)			
7 (23)			

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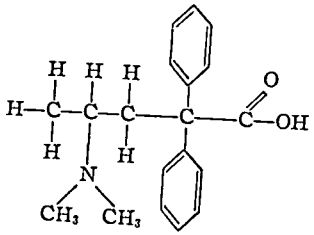

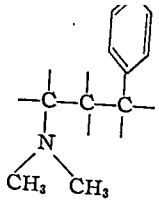

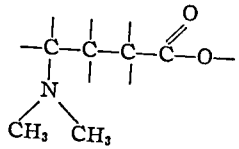

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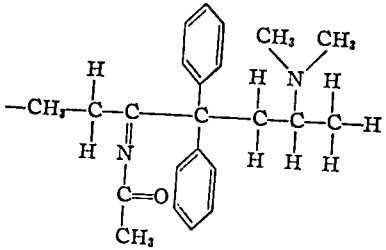

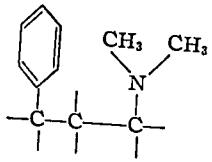

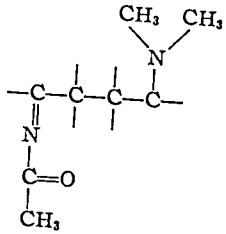

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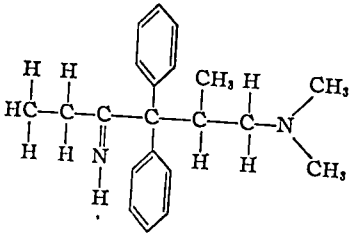

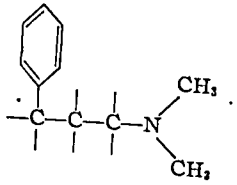

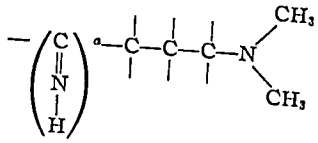

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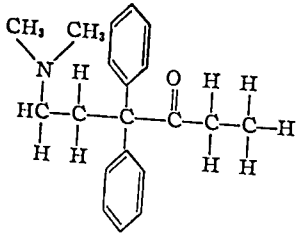
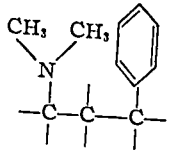
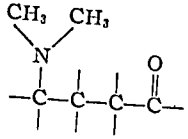


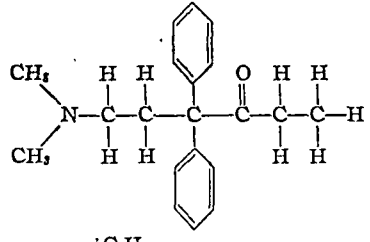
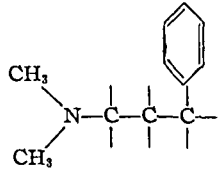
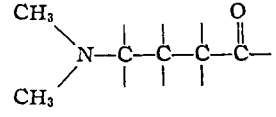
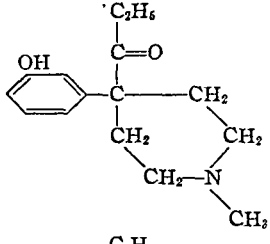
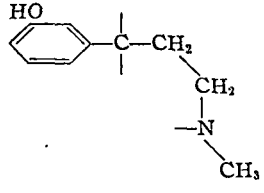
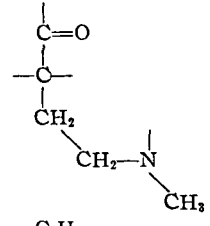
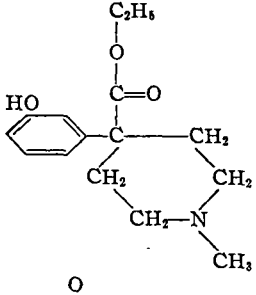
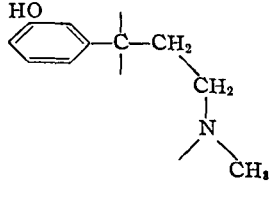
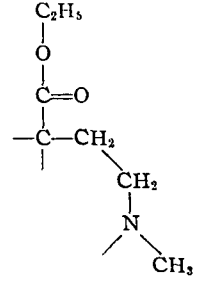
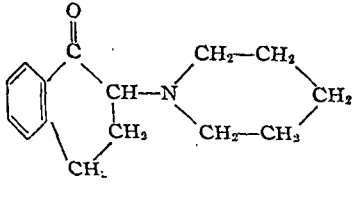
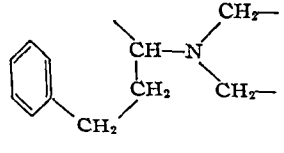
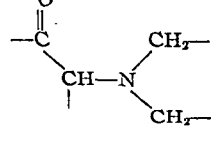




[a] From the organic chemical point of view $\diagup C = N{-}H$ is analogous to $\diagup C = O$ just as $-NH_2$ is to $-OH$.

(*Cont'd on p. 78*)

TABLE I.—(*Cont'd from p. 77*)

No. and Reference	Drug Structure	Sympathomimetic Moiety	Parasympathomimetic Moiety
12 (11)			
13 (11)			
14 (9)			
15 (13)			

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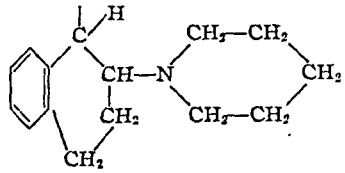

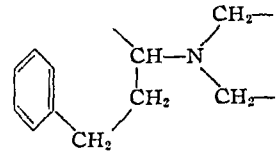

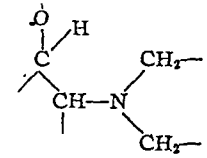

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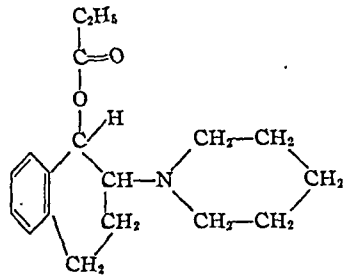

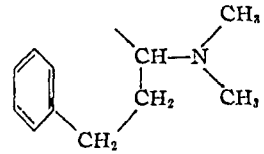

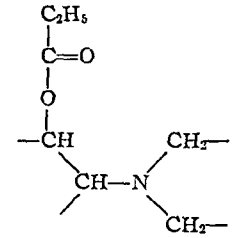

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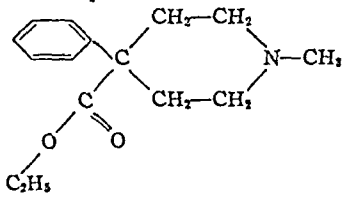

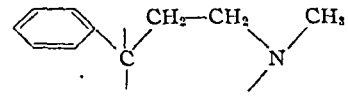

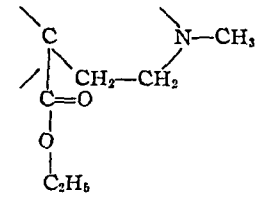

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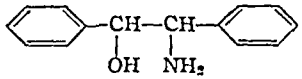
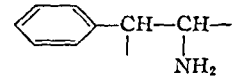
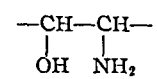
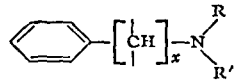


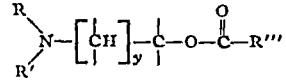




Moieties of the phenyl-alkyl-ω-amine type are termed sympathomimetic:



In general, the optimum activity of these amines with regard to sympathomimetic effect is obtained when $x = 2$ to 4.
Moieties of the tertiary amino alkyl type with terminal oxygen groups of the ether, ketone, or alcohol type are termed parasympathomimetic.



In general, the optimum activity of these amines with regard to parasympathetic effect is obtained when $y = 1$ to 3 and when *both* ketonic and ether oxygen (as in an ester) are present. When ketonic, ether, or alcoholic oxygen alone are present the parasympathomimetic effect is decreased but qualitatively similar to that of the more active analogs (17).

analogs and suggests an explanation of their parasympathomimetic type action. (See Table II.) In a similar manner, essential distances appear to function in the sympathomimetic moieties of the analgetic drug structures. Further support of these ideas is gained from analgetic tests using the Wolff-Hardy-Goodell (27) technique with white rats. Such tests show combinations of sympathomimetic agents and parasympathomimetic agents to act additively and possibly synergistically in certain dosage ranges. These effects are illustrated in Table III.

Finally, it is suggested that the analgetic action of the morphine type agents may be traceable to the presence of both sympathomimetic and parasympathomimetic moieties in the same molecule which act physiologically through a peripheral analgetic mechanism. The physiological mechanism may be a peripheral perineural vasoconstrictor effect as outlined by Gross (1,4). Recent evidence (6) has been reported to the effect that adrenectomized rats show less analgesia from morphine than normal rats and that this loss in morphine analgesia is due to the loss of epinephrine output by the adrenal medulla. These investigations emphasize the possible importance of peripheryl mechanisms in analgesia and support the notions advanced by Buscaino and Pero as to the pre-eminence of autonomic components in the analgetic process.

The parasympathomimetic portion of the analgetic molecule may be active in stimulating the output of epinephrine by the adrenal gland (3, 7), either by its direct action or through the *inhibitory action upon cholinesterase, and the* sympathomimetic portion of the molecule may enhance the peripheral perineural vasoconstrictor response of the epinephrine by either its direct action or by preserving epinephrine. Thus, a sympathomimetic moiety alone will exhibit some analgetic effect but when this is coupled with the epinephrine output, through stimulation of the adrenal gland by the parasympathomimetic moiety, the total analgesic effect will be maximal. Another important consideration is the fact that the powerful analgesics show a sedative effect reaching a peak which is not simultaneous with the peak analgetic response, and this sedative effect must be an expression of the constitutional relationships involved in this group of substances. The fact that analgesia reaches its peak at a different time than sedation may be explainable on the basis that the sites of analgetic drug action are different from the sites of sedative action. Indeed, the diphasic-stimulant and depressant properties (as in the cat or dog) of the powerful morphine analogs may be correlated with the relative sensitivity of the central

TABLE II.

Compound No.	x	Sympathomimetic Distance[a]	y	Parasympathomimetic Distance[b]	
1	2	5.0	2	6.0	
2	3	5.5	3	7.0	
3	3	5.5	2	7.0	C=O
				5.0	—O—
4	3	5.5	2	7.0	C=
				5.0	—O—
5	3	6.3	3	8.0	
6	3	6.3	3	8.0	
7	3	6.3	3–2	5.0	—O—
				8.0	=O
8	3	6.5	2	7.5	
9	3	6.5	3	9.0	
10	3	6.3	3	8.5	
11	3	6.3	2	8.0	
12	3	6.0	3	8.0	
13	3	5.5	3	8.0	
14	3	5.5	3	8.0	
15	3	6.0	1	5.5	
16	3	6.0	1	5.5	
17	3	6.0	1	5.5	—O—
				7.5	=O
18	3	5.5	2	7.5	
19	2	4.5	1	5.0	

[a] Sympathomimetic Distance denotes the average maximum distance between the first carbon on any alkyl group of the amino nitrogen atom and the aromatic carbon joining the ring to the alkyl carbon chain.

[illegible] Distance denotes the average [illegible] the first carbon on any alkyl [illegible] atom and the terminal oxygen atom as computed by measurements using Fisher-Hirschfelder-Taylor Models (1 cm. = 1 Å.).

TABLE III.—MEAN ± STANDARD ERROR OF THE RISE IN ANALGETIC RESPONSE

Drug[a]	Dosage in Mg./Kg.	10 Min. After Injection	20 Min. After Injection
Ephedrine	4	2.7 ± 0.87	2.1 ± 0.42
Doryl	0.5	6.7 ± 1.56	3.4 ± 1.17
Ephedrine	2	−0.08 ± 0.42	−1.08 ± 0.42
Doryl	0.25	1.21 ± 0.65	2.34 ± 0.44
Actual effect of:			
Doryl	0.25		
Ephedrine	2	6.5 ± 1.25	3.5 ± 0.89
Theoretical effect of:			
Doryl	0.25	1.13 ± 0.76	2.26 ± 0.6
Ephedrine	2		

[a] All drugs dissolved in saline were given by intraperitoneal injection.
The rise in analgetic response is expressed as the mean increase in time (seconds) required for the reflex tail twitch of a given group of rats before and after injection of the drug using the Wolff-Hardy-Goodell technique.

nervous system of these animals to the stimulant and depressant properties of the moieties embedded within the molecular structures.

It may be pointed out that in all such attempts to correlate chemical constitution with pharmacodynamic action the most desirable results are those which suggest an immediate translation of the chemical constitutional considerations into physiological terms. Thus, with the analgesics, consideration of "whole-molecule" properties gives little insight into the connection between their chemistry and pharmacology while recognition of physiologically active moieties within the whole structure gives a plausible interpretation of their actions with reference to the autonomic nervous system. Once these essential moieties are recognized one may approach the synthesis of new agents possessing the given activity with an almost infinite range of possible variations so long as the essential moiety relationships are maintained. Changes in the constitution of these molecules which increase or decrease their lipoid solubility, and penetrability into nervous tissue and cells will, of course, also effect quantitative changes in their activity. Further, the introduction of steric factors and groups which modify the electronic properties of the physiologically active moieties will cause quantitative changes in activity. Tentatively one might suggest that the ideal molecule for a given type of pharmacodynamic activity would be relatively inert with the exception of the essential moieties embedded in it and that any modifications that would be introduced should enhance the ease with which the molecule may reach its given site of action.

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The Quantitative Estimation of Sulfoxylate-Sulfur*

By C. K. BANKS, JACQUELINE D. MITULSKI, D. F. WALKER, and L. M. WHEELER

Existing analytical procedures for the determination of sulfoxylate-sulfur are inadequate for several compounds of pharmaceutical importance. Methods were developed for the determination and differentiation of this class of sulfur compounds.

THE increased use of sodium formaldehyde-sulfoxylate as a solubilizing agent for medicinal compounds containing aromatic amine groups has indicated the need for a method of estimating sulfoxylate-sulfur and related sulfur in such compounds. Not only is a method needed for the methylsulfoxylate linkage but also for its partially oxidized products which may not be identical with the methanesulfonate products, $-NHCH_2SO_3^-$.

Sodium formaldehyde sulfoxylate had been assayed with methylene blue in hot dilute acetic acid under nitrogen (1, 2), with ammoniacal copper sulfate (3–5) under hydrogen at 55°, and with iodine in "neutral" solution (2, 4). These

* Received July 22, 1948, from the Research Laboratories of Parke, Davis and Co., Detroit, Mich.

three procedures did not give equivalent results (2, 4). Attempts have also been made to analyze methylsulfoxylate derivatives of aromatic amines (1, 6–12). Elvove (8) suggested an indigotindisulfonic acid method for neoarsphenamine but Dyke and King (12) showed that the results obtained by this method were high. The methylene blue titration cited by Freedman (1) was found to yield values from 71 per cent to 90 per cent of the expected (12). Iodine has been used widely (6–12) but the severe criticism of this method by Freedman (1) and the data of Dyke and King (12) indicated that the results were not quantitative.

Since none of the methods as described appeared to be accurate, it was decided to investigate the iodine method. This choice was dictated by the larger general knowledge of iodimetry as well as avoidance of inert atmospheres and increased temperatures. Sodium formaldehyde sulfoxylate when titrated with iodine appears to react stoichiometrically according to Equation I, provided a few drops of acetic acid are added prior to titration. If the acetic acid is not added, the results may be somewhat irregular.

$$HOCH_2{\cdot}SO_2Na + 2I_2 + 2H_2O \xrightarrow{[H^+]} CH_2O + NaHSO_4 + 4HI \quad (I)$$

Titration of arylaminomethylsulfoxylates, however, was found to be incomplete, approximately 90 per cent of the stoichiometric amount of iodine being used as had been noted previously (12). Since a trace of acid appeared to be necessary for the titration of inorganic sulfoxylates, a slight amount of acetic acid was added prior to titration. This resulted in an increased consumption of iodine and the end point became more vague. When hydrochloric acid was used the titration became nearly quantitative but the end point was extremely unstable. Since the last part of the titration appeared to be due to a reaction having a slow velocity, the organic sulfoxylates were dissolved in water, acidified with acetic acid and then added to an excess of iodine in an iodine flask, acidified with hydrochloric acid and allowed to stand for varying periods of time. Back-titration with thiosulfate indicated that the maximum amount of iodine was used after forty minutes and the value was unchanged up to several hours. Furthermore, the quantity of iodine used was stoichiometric according to Equation II.

$$RNHCH_2{\cdot}SO_2Na + 2I_2 + 3H_2O \xrightarrow{[H^+]} RNHCH_2OH + NaHSO_4 + 4HI \quad (II)$$

The procedure was verified on a sample of known purity. This procedure was then used on about forty different samples, representing a number of arylaminomethylsulfoxylates. A total of about seventy-five duplicate assays were made. The statistical precision of the method was determined by the variations of the individual assays from their average in parts per thousand.

Experiments with arylaminomethanesulfonates indicated no absorption of iodine. While the procedure indicated a small variable blank, no blank correction was made except on hot days when the maintenance of standard solutions was difficult. The thiosulfate equivalence of the iodine was determined for each group of assays. A definite excess of iodine had to be present to give duplicable results.

Since arylaminomethanesulfoxylates are extremely susceptible to air oxidation, sulfoxylate oxidation products were also titrated. Some iodine was utilized by these compounds, raising the question as to whether this was due to an intermediate compound or to a mixture of oxidized and unoxidized compounds. The literature on this subject is conflicting. It was noted that Heyl and Greer (2) had claimed that certain lots of sodium formaldehydesulfoxylate after titration with iodine in unbuffered solution had absorbed a further quantity of iodine in bicarbonate buffers. A similar variation was noted with our arylaminomethylsulfoxylates. This end point was extremely variable. When the samples, however, were added to excess iodine and made sufficiently alkaline to bleach the solution, and then allowed to stand an hour acidification returned any unused iodine and the stoichiometry appeared to fit Equations III and IV.

$$HOCH_2{\cdot}SO_2Na + 3I_2 + 3H_2O \xrightarrow{[OH^-]} HCOOH + NaHSO_4 + 6HI \quad (III)$$

$$RNHCH_2{\cdot}SO_2Na + 3I_2 + 3H_2O \xrightarrow{[OH^-]} HCONHR + NaHSO_4 + 6HI \quad (IV)$$

Arylaminomethanesulfonates, when treated similarly, absorbed no iodine. Oxidation products of arylaminomethylsulfoxylates also reacted with alkaline iodine, utilizing a different quantity of iodine under alkaline conditions from that used in acids.

EXPERIMENTAL

Standard solutions were prepared according to Kolthoff and Sandell (13). Iodine, ca. 0.05 *N*, was checked for possible iodate contamination before each use (14). Sodium thiosulfate, ca. 0.05 *N*, was standardized each day.

Acid Sulfoxylate Determinations.—Accurately weigh samples of the compound to be assayed so as to have not more than 7 to 8 mg. of sulfoxylate-sulfur per sample. Accurately transfer 30-ml. quantities of standard iodine (ca. 0.05 *N*) to iodine flasks. Dissolve the samples in distilled water (ca. 10 ml.) using a drop of *N* sodium hydroxide if necessary. Acidify the samples with 2 ml. of glacial acetic acid (methylenesulfoxylates and methylsulfonates do not precipitate at this point) and transfer quantitatively to the iodine flasks. Add 3 ml. of concentrated hydrochloric acid, stopper, mix thoroughly and allow to stand an hour at room temperature. Titrate the excess iodine with standard sodium thiosulfate (ca. 0.05 *N*) using starch solution at the end point. Each ml. of 0.05 *N* iodine is equivalent to 0.0004004 Gm. of sulfoxylate sulfur. The complete calculation is given by:

$$\%S \text{ (sulfoxylate)} = \frac{[\text{Ml. } (I_2) \times N(I_2) - \text{ml. } (S_2O_3^{=}) \times N(S_2O_3^{=})] \times 0.03206}{\text{Weight of sample} \times 4}$$

If 0.1 *N* solutions are used, the sample may contain up to 15 mg. of sulfoxylate sulfur.

Alkaline Sulfoxylate Determinations.—Accurately weigh samples of the compound to be assayed so as to have not more than 8 mg. of sulfoxylate sulfur per sample. Accurately transfer 50 ml. of standard iodine (ca. 0.05 *N*) to iodine flasks. Dissolve the samples as in the acid determination and transfer quantitatively to the iodine flasks. Add 0.1 *N* sodium hydroxide until the iodine color is bleached. Allow to stand an hour at room temperature, acidify with an equivalent of concentrated hydrochloric acid and 3 ml. excess, allow to stand fifteen minutes and titrate the excess iodine with standard sodium thiosulfate solution (ca. 0.05 *N*) using starch indicator. Each ml. of 0.05 *N* iodine is equivalent to 0.0002672 Gm. of sulfoxylate sulfur. The complete calculation is given by:

$$\%S \text{ (sulfoxylate)} = \frac{[\text{Ml. } (I_2) \times N(I_2) - \text{ml. } (S_2O_3^{=}) \times N(S_2O_3^{=})] \times 0.03206}{\text{Weight of sample} \times 6}$$

Disodium Dimethylenesulfoxylate of 4,4′-Diaminodiphenylsulfone.—A sample of the disulfoxylate derivative of 4,4′-diaminodiphenylsulfone of known purity was assayed as described. The results are shown in Table I.

Anal.—Calcd. for $C_{14}H_{14}O_6N_2S_3Na_2$: S, 14.32%. Found: S (sulfoxylate-acid) 14.30% (av. 8); (sulfoxylate-alkaline) 14.26% (av. 6).

TABLE I.—ESTIMATION OF SULFOXYLATE SULFUR IN THE DISODIUM DIMETHYLENESULFOXYLATE OF 4,4′-DIAMINODIPHENYLSULFONE

Per Cent Sulfoxylate Sulfur	
Acid Detn.	Alkaline Detn.
14.20	14.32
14.31	14.44
14.12	14.20
14.42	14.17
14.28	14.28
14.34	14.16
14.38	...
14.27	...

Sodium Formaldehydesulfoxylate.—Samples of reagent grade sodium formaldehydesulfoxylate (Merck) were assayed by the acid and alkaline methods and by direct titration in the presence of acetic acid. The results are shown in Table II.

TABLE II.—ESTIMATION OF SULFOXYLATE SULFUR IN SODIUM FORMALDEHYDESULFOXYLATE

	Per Cent Sodium Formaldehydesulfoxylate		
	Acid Detn.	Alkaline Detn.	Direct Iodine Detn.
	99.76	99.53	99.67
	99.75	99.30	99.77
	99.89	99.71	99.44
	99.90	99.77	99.80
Averages	99.83	99.58	99.67

While the above averages are not conclusive checks, the allowable variation in the methods as shown below indicates the above values as obtained by the acid and alkaline methods to be not significantly different from the direct titration value.

Deviation of Assays.—A group of 77 assays on 20 samples was listed by deviation from the averages in parts per thousand. The data when grouped in unit parts per thousand were analyzed statistically. (Table III.)

TABLE III.—STATISTICAL ANALYSIS OF ASSAY DEVIATIONS

Deviation, P. P. T.	Frequency	$N(d')^2$
0–1	15	3.75
1–2	10	22.50
2–3	12	75.00
3–4	11	134.75
4–5	8	162.00
5–6	7	211.75
6–7	5	211.25
7–8	4	225.00
8–9	2	144.50
9–10	2	180.50
10–11	1	110.25
Total	77	1481.25
Variance		19.4901
Standard Deviation (single assay)		4.4
Standard Deviation (two assays)		3.1
Standard Deviation (four assays)		2.2
99% limits (single value)		11.5 p. p. t.
99% limits (av. two)		8.2 p. p. t.
99% limits (av. four)		5.8 p. p. t.

SUMMARY

An assay method for determining the per cent sulfoxylate sulfur in arylaminomethylsulfoxylates involving acidic iodine oxidation has been presented. The precision of single assay values by the method is 11.5 parts per thousand at 99 per cent probability. While the precision is not exceptional, the accuracy of multiple determinations would appear to be of the order of 6 parts per

thousand. A check assay for methylenesulfoxylate linkages has also been presented, utilizing both acid and alkaline oxidation by iodine.

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Hydrogenolysis as a Method for Determining Chlorguanide (Paludrine)*

By ROBERT C. SHULTZ

Chlorguanide may be determined quantitatively by hydrogenolysis in acid solution with zinc amalgam to form a primary aromatic amine which is subsequently measured colorimetrically by application of a modified Bratton-Marshall reaction.

THROUGH the use of zinc amalgam in an acid medium and an efficient shaking machine, chlorguanide, N_1-chlorophenyl-N_5-isopropyl biguanide, can be cleaved quantitatively to form a primary aromatic amine, probably *p*-chloroaniline or aniline. The amine can be measured conveniently by diazotization followed by coupling to form an azo dye according to a conventional modification of the original Bratton-Marshall reaction (1). Spinks and Tottey describe a method for the determination of chlorguanide in blood and tissue in which *p*-chloroaniline is formed by the hydrolysis of chlorguanide in an autoclave for a period of four hours (2, 3). In this procedure the sample must be sealed in an ampul. Zinc and zinc amalgam have been used to split pteroylglutamic acid by a similar procedure described by Hutchings, *et al.* (4).

EXPERIMENTAL

A solution of a commercial sample of chlorguanide in 0.5 *N* hydrochloric acid (supplied by E. I. du Pont de Nemours and Company, Inc.) was analyzed by the procedure described in the present communication, using concentrations of from 1 to 6 mg. in each 10 ml. of solution. A linear relationship between concentration and color production was found (Fig. 1). In order to determine the optimal time required for complete cleavage, the amine formed was measured at ten-minute intervals during an eighty-minute period of shaking with zinc amalgam. No further increase could be detected after sixty minutes (Fig. 2).

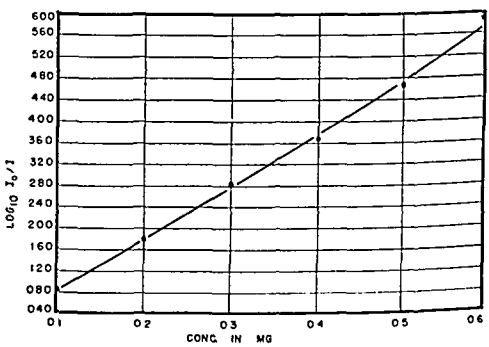

Figure 1.

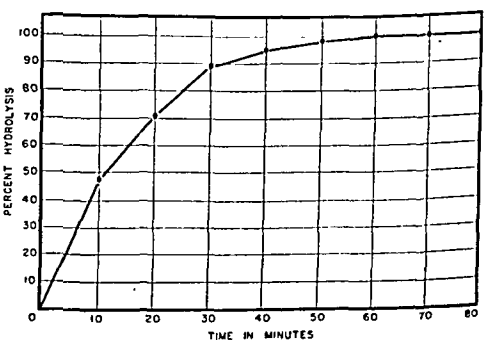

Figure 2.

* Received August 11, 1948, from the Pharmaceutical Research Laboratories, Medical Research Division, Sharp & Dohme, Inc., Glenolden, Pa.

For the work in this laboratory a "Precision" Equipoise heavy-duty shaker No. 5855 was employed. Some variation in the time required for quantitative cleavage may result, depending on the relative efficiency of the shaker used. The procedure, as developed particularly for application to drug products, follows.

Reagents—Zinc amalgam. To 20 ml. of mercury add 10 Gm. of mossy zinc. Heat with stirring to 150° until the zinc is dissolved.

0.1% aqueous sodium nitrite.

0.5% aqueous ammonium sulfamate.

0.1% freshly prepared aqueous *N*-1(1-naphthyl) ethylene diamine dihydrochloride.

0.5 *N* hydrochloric acid.

Chlorguanide standard solution: 4 mg. per 100 ml. in 0.5 *N* HCl.

Procedure.—Prepare an extractive or solution of the sample and adjust it to contain approximately 0.4 mg. of chlorguanide in each 10 ml. of 0.5 *N* hydrochloric acid. Transfer 50–60 ml. of this solution to a bottle, add 5 ml. of the zinc amalgam and stopper tightly. Shake mechanically for one hour. Transfer exactly 10 ml. of the solution reduced in this way to a 100-ml. volumetric flask; add 5 ml. of *N* HCl and 35 ml. of H_2O. Add as directed: 5.0 ml. of 0.1% sodium nitrite—wait two minutes; 5.0 ml. of 0.5% ammonium sulfamate—wait three minutes; and 5.0 ml. of 0.1% *N*-(1-naphthyl) ethylene diamine dihydrochloride. Wait ten minutes and dilute to 100 ml. with 0.15 *N* hydrochloric acid. Mix well and measure the transmission in a Beckman spectrophotometer at a wave length of 550 mμ, or in a suitable colorimeter. Calculate the extinction ($\log_{10} I_0/I$) and compare it with that found for a 10-ml. portion of the standard chlorguanide solution run in the same way and at the same time.

SUMMARY

A method is described wherein chlorguanide is determined through cleavage by hydrogenolysis in acid solution with zinc amalgam to yield a primary aromatic amine which is subsequently measured colorimetrically through the application of a modified Bratton-Marshall reaction.

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Microtoxicology. IV. The Identification of Antihistaminic Drugs of the Thenyl Series*

By THOMAS J. HALEY† and GEORGE L. KEENAN‡,§

The optical crystallographic properties of antihistaminic drugs of the thenyl series have been determined and are recorded in this report. Other means of identification and differentiation involving the use of six common colorimetric alkaloidal reagents are described. The crystallographic properties described offer the best means of identification.

THE extensive use of such drugs as Benadryl Pyribenzamine in the treatment of allergenic conditions has led to the synthesis and therapeutic use of an increasing number of antihistaminic agents. Among the latest drugs of this group are Thenylene or Histadyl (N,N-dimethyl-N′-(2-pyridyl)-N′-(2-thenyl)-ethylenediamine) and its halogenated derivatives Chlorothen (N,N - dimethyl - N′ - (2 - pyridyl) - N′ - (5-chloro - 2 - thenyl) ethylene diamine) and Bromothen (N,N-dimethyl-N′-(2-pyridyl)-N′-(5-bromo-2-thenyl) ethylenediamine)(1, 3, 13). Table I lists the physical constants for these compounds.

TABLE I.—PHYSICAL CONSTANTS OF THE THENYL ANTIHISTAMINES

Compound	Boiling Point ° C.	Mm.	Melting Point, ° C.	Reference
Histadyl base	173–175	3	.....	14
Thenylene HCl	.....	..	161–162	..
Methoiodide	.....	..	156–157 dec.	..
Chlorothen				
Base	155–156	1	.....	1
HCl	.....	..	106–108	..
(Tagathen)				
Citrate	.....	..	116–118	..
Bromothen				
Base	173–175	1	.....	1
HCl	.....	..	124–126	..

* Received June 28, 1948, from the Medical School, University of California at Los Angeles, Calif.
† Medical School, University of California at Los Angeles.
‡ Strongsville, Ohio, and Baldwin-Wallace College, Berea, Ohio.
§ The generous supplies of Histadyl (Thenylene) were furnished by Eli Lilly and Co. and Abbott Laboratories, and the Chlorothen and Bromothen by Lederle Laboratories.

The pharmacological activity (2, 7, 8, 10) and therapeutic effects (4,9) of these compounds have been described but as yet no simple means for their identification has been published. This investigation was undertaken to determine the reactivity of these Thenyl antihistaminics with several simple alkaloidal precipitants and color reactions to see if such tests could serve as a means of identification. Further, comparison was made with the color reactions already recorded in the literature to prevent confusion and misinterpretation of the results obtained

EXPERIMENTAL

The reagents were prepared as directed by Thienes and Haley (12). The compounds were tested by placing one drop of a reagent on a microscope slide and adding about one milligram of the drug to it. The results of this addition were observed for about thirty minutes and changes taking place were recorded. In the case of the acidic colorimetric reagents the end point was a charring of the drug, but only the colors appearing before this were used for identification. Table II gives the results of precipitation and colorimetric tests.

Inasmuch as the precipitation and colorimetric tests were almost identical for each of the Thenyl compounds, the optical crystallographic properties of pure compounds were studied.

Histadyl.—In ordinary light, the habit is rather thick plates, hexagonal in outline, and readily breaking up into irregular fragments.

Refractive indices: $\alpha = 1.588$, $\beta = 1.654$, $\gamma = >1.695$, but <1.734; all ± 0.002. α and β are most frequently observed.

In parallel polarized light (crossed nicols): Many of the fragments do not extinguish sharply with crossed nicols.

In convergent polarized light (crossed nicols): Partial biaxial interference figures frequently shown with one optic axis in the microscopic field. Optic sign: (−).

Chlorothen Hydrochloride.—In ordinary light, the substance consists of massive fragments, some of which are rectangular in shape.

Refractive indices: $\alpha = 1.553$ (commonly shown), $\beta = 1.625$ (not common), $\gamma = >1.734$; both ± 0.002.

In parallel polarized light (crossed nicols): Many fragments do not extinguish sharply with crossed nicols.

In convergent polarized light (crossed nicols): Optic axis figures occasionally shown, and frequently inclined to the axis.

Chlorothen Citrate.—The substance occurs as very minute fragments, in ordinary light, consisting of plates and shreds. The plates frequently are rhombic in outline, and the shreds consist of plates tipped on edge.

Refractive indices: $\alpha = 1.583$, $\beta = 1.603$, $\gamma = 1.645$; all ± 0.002. β and γ frequently shown on plates on edge.

In parallel polarized light (crossed nicols): Many of the fragments do not extinguish sharply with crossed nicols.

In convergent polarized light (crossed nicols): Optic axis figures can be observed, but they are the exception rather than the rule, due to the small size of the plates.

Bromothen Hydrochloride.—In ordinary light, the substance is rod-like in habit, the rods being very small.

Refractive indices: $\alpha = 1.617$ (common), $\beta = 1.654$, $\gamma = 1.734$ (also common); all ± 0.002.

In parallel polarized light (crossed nicols): The extinction usually is inclined on the rods and the sign of elongation is positive. The rods, however, do not extinguish sharply.

In convergent polarized light (crossed nicols): Partial biaxial interference figures showing the optic axis inclined occasionally are shown, but rods are too small to show ideal figures.

The photomicrographs of the pure compounds were taken with a Visicam photographic attachment

TABLE II.—REACTION OF THENYL ANTIHISTAMINES WITH VARIOUS REAGENTS

Reagent	Histadyl HCl	Bromothen HCl	Chlorothen HCl	Tagathen Chlorothen Citrate
Conc. H_2SO_4	Burnt orange changing to blood red, finally deep purple	Magenta, then deep purple	Magenta, then deep purple	Magenta, then deep purple
Conc. HNO_3	Purple-pink changing to brown	Orange, changing to lemon yellow	Yellow	Orange-red
Mandelin's	Burnt orange	Deep reddish orange	Deep reddish orange	Deep reddish orange
Marquis	Orange-brown changing to purplish pink	Bright red	Carmine red with a slight purple cast	Brilliant magenta
Fröhde's	Dark brown with black streaks	Deep reddish purple	Deep reddish purple	Deep reddish purple
Buckingham's	Brown, then black	Deep reddish purple	Deep reddish purple	Deep reddish purple
Chloroplatinic acid	Branched bundles of rods in feather-like agglomerates	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate
Chlorauric acid	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate
Picric acid saturated aqueous soln.	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate

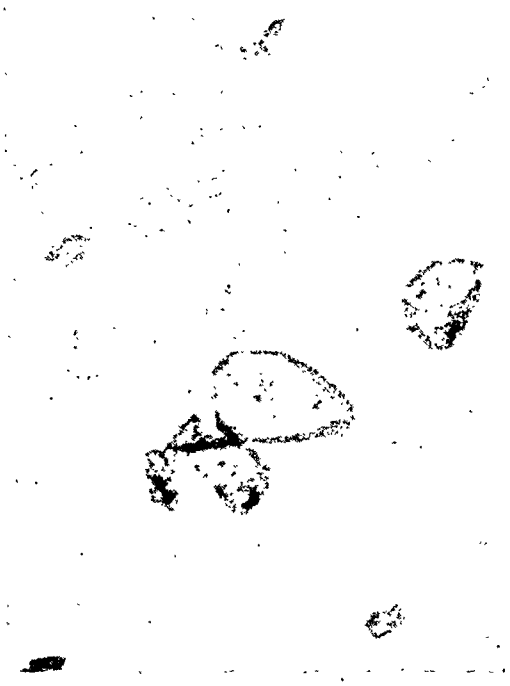

Fig. 1.—Thenylene. Magnification 115×.

on a Band L binocular microscope using the 16-mm. objective and the 10× eyepiece. All of the compounds have distinctive crystalline structure which may be used as a further means for their identification and differentiation. See Figs. 1, 2, 3, and 4 for the photomicrographs.

DISCUSSION

Although papaverine, narceine, narcotine, physostigmine, codeine, morphine, strychnine, thebaine, and veratrine give various shades of yellow, yellow-red, or orange with nitric acid (11), the color reactions of these alkaloids with concentrated sulfuric acid (11) are so different from those of the Thenyl antihistamines that no difficulty would be encountered in their differentiation. With Mandelin's reagent (11), cinchonine, cocaine, pilocarpine, quinine, solanine, thebaine, and veratrine produce colors similar to those given by the Thenyl compounds, but here again comparison of their reactions to other colorimetric reagents would differentiate the compounds from each other. The colors produced with Marquis' reagent (11) are different from any of those recorded in the literature and thus this reagent is the best one for differentiation and identification of Thenyl compounds not only from each other but from other drugs and alkaloids. Both aconitine and veratrine give a brownish color with Buckingham's reagent (11) but the final color is considerably different from the color obtained with Histadyl. The other Thenyl compounds give the same colorimetric reaction, thus preventing their differentiation with this reagent. However, the color does differ from any previously reported for other compounds so that the reaction is of value in

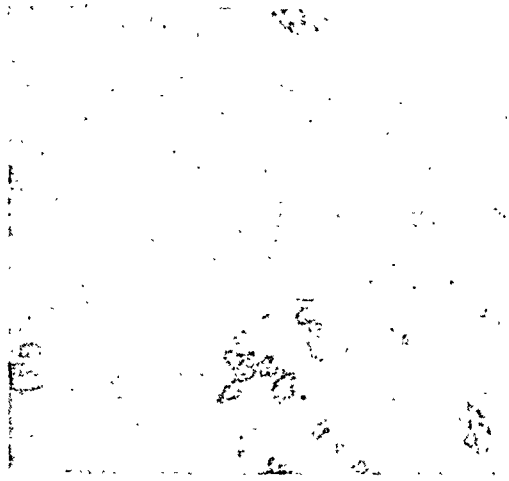

Fig. 2.—Chlorothen. Magnification 75×.

differentiating these antihistaminics from other compounds or alkaloids. Aconitine, berberine, brucine, colcynthin, digitalin, morphine, narceine, and solanine give varying degrees of brownish coloration with Fröhde's reagent (11) but all of these alkaloids may be differentiated from Histadyl by their preliminary or final colors. The other Thenyl compounds cannot be differentiated from each other by this test but can be differentiated from brucine, colcynthin, curarine, digitalin, morphine, nicotine, papaverine, thebaine, or veratrine. These latter compounds show changing colors while the

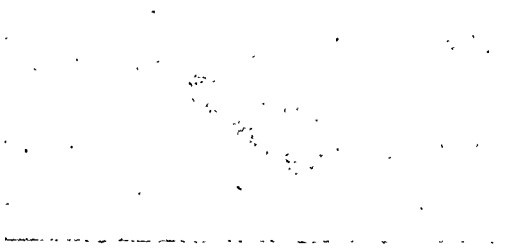

Fig. 3.—Tagathen. Magnification 265×.

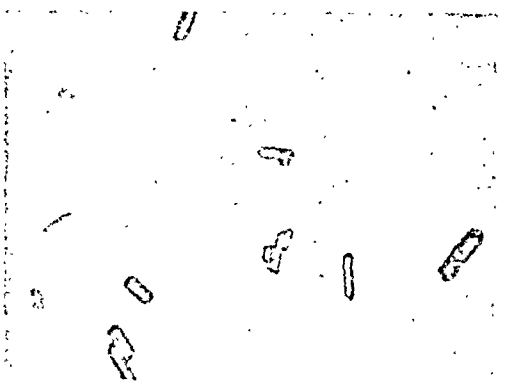

Fig. 4.—Bromothen. Magnification 65×.

Thenyl antihistaminics develop only one stable color. Benadryl and Pyribenzamine both produce colors with the color reagents used with Thenyl compounds, but the color reactions are so different that no confusion would result when comparisons are made (5).

Keenan (6) has described definite crystalline precipitates for both Benadryl and Pyribenzamine with chloroplatinic acid but none with chlorauric acid. The crystalline precipitate formed when Histadyl is mixed with chloroplatinic acid differs greatly from those formed by both Benadryl and Pyribenzamine. The other Thenyl antihistaminics could be identified by their reactions with any of the precipitants used because amorphous precipitates were formed in all cases.

SUMMARY

A means for the identification and differentiation of the Thenyl antihistaminic drugs has been described. The six common alkaloidal colorimetric reagents used gave better results than three precipitation reagents. However, the optical crystallographic properties described offer the best means for the identification of the Thenyl antihistaminics.

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The Bacteriological Aspects of Deionized Water*

By PHILIP C. EISMAN, F. C. KULL, and R. L. MAYER

A bacteriological study was made of demineralizing units containing synthetic anion and cation exchange resins for the production of water with low mineral content. The conditions studied were: the chemical purity of the effluent, intervals of time elapsing between successive operations of the deionizing units, and the frequency of resin regeneration. The results obtained indicate that there exists a wide variation in the bacterial content of deionized water which is conditioned by the manner in which these units are operated and that the bacterial content of the effluent bears no relationship to its chemical purity.

THE DISTILLED water ordinarily used in chemical and bacteriological laboratories and in the pharmaceutical, food, and related industries is being replaced to a considerable extent by the so-called demineralized or deionized water prepared by the action of synthetic ionic exchange resins. Because of the nature of these resins, deionizing units are also being employed in a variety of operations such as the removal of ionized impurities from solutions of nonelectrolytes (1) and for the separation and purification of ionizable biologicals such as vitamins (2) and amino acids (3).

Deionized water, when properly prepared, corresponds to distilled water with respect to the absence of inorganic salts (4). Moreover Harrisson and co-workers (5) have reported that resinous exchanger systems would produce water equal in quality to that required by U. S. P. specifications and that the pyrogen content was unaffected by the exchange process. But contrary to distilled water, it may contain significant amounts of organic matter, depending upon the nature of the raw water entering the demineralizing unit (2). Furthermore, distilled water by the very nature of its preparation is sterile at the time of its production and only the conditions of its storage and handling determine the nature and extent of its subsequent bacterial population. Observations made in our laboratory indicated that, unlike distilled water, deionized water was only occasionally sterile and often grossly contaminated at the time of production. Since the use of deionized water in various laboratories and

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manufacturing processes makes it necessary that it be relatively free of microorganisms, a bacteriological study of this type of water was undertaken.

Since many desirable features are associated with the use of demineralizing units, it may be expected that a more general, industry-wide acceptance of this process will take place in the near future. Experimental data yielding information regarding the bacterial aspects of the water so produced are therefore not only of timely interest, but would indicate those conditions of operation yielding a product of low bacterial content.

In the deionizing units such as that constructed by the American Cyanamid Company (Filt-R-Stil Demineralizers) and which have furnished the water investigated during the course of the present work, raw water is subjected to the successive action of synthetic cation and anion resins and is as a result almost completely demineralized. As shown in Fig. 1, which is adapted from data obtained from the American Cyanamid Company, each of the units consists of four resin beds arranged in series and alternately containing cation and anion synthetic resins. The end product or effluent, which has a *p*H value usually between 6.5 and 7.5, contains less than 2 parts per million of ionizable salt (less than the 2 mg. of salt residue per liter of water) and is therefore chemically as pure as properly prepared distilled water in so far as ionizable inorganic residues are concerned.

The amount of raw water a deionizing unit will efficiently process depends upon the ionizable salt content of the water. A Barnstead purity meter expressing values in terms of ionizable salt content, indicates the actual efficiency of the unit at any given moment. Supplementing the purity meter is a system of green and red indicator light bulbs which operate according to the electrical conductivity of the water issuing from the unit. A green light indicates an effluent containing a mineral content below a predetermined maximum, while a red light denotes an excessive mineral content. When the capacity of the resins to remove ions has been exhausted as shown by the purity meter or the red light, it is necessary to halt the deionizing process and regenerate both the cation and anion exchange resins. The frequency between two successive regenerations is dependent upon the volume

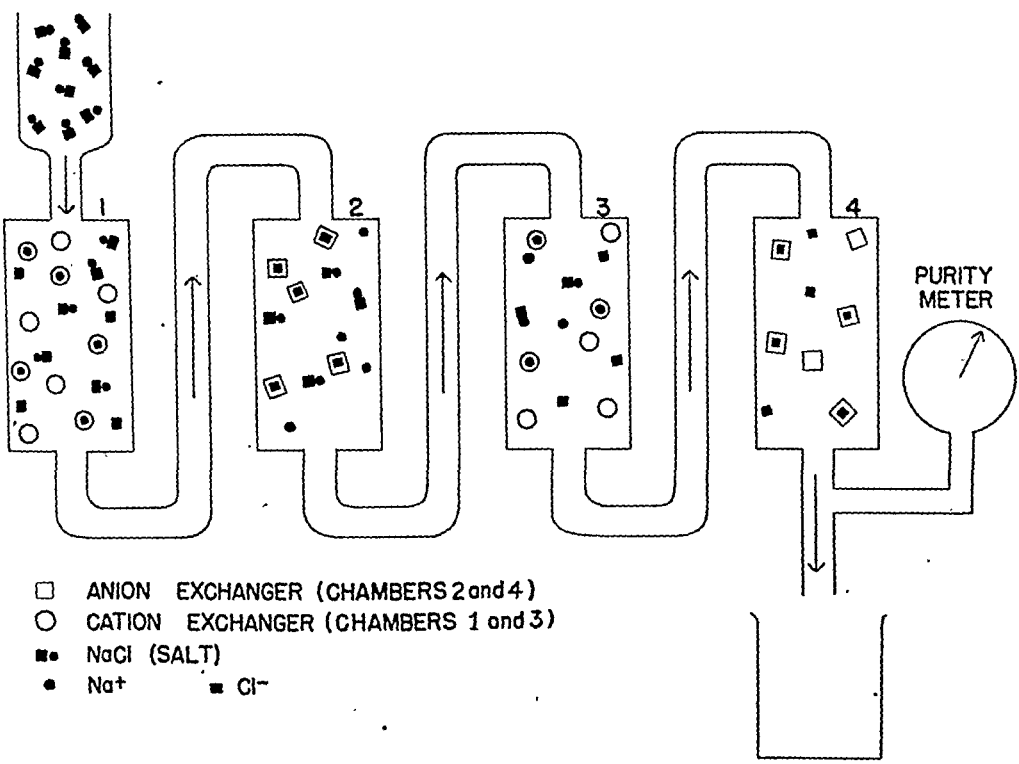

Fig. 1.—A deionizing unit.

of water processed and is also conditioned by the amount of dissolved mineral matter contained in it. It is therefore possible that a unit with a capacity for treating 1500 gallons may require regeneration only after several weeks of use when relatively small amounts of water with a low original mineral content are occasionally required, or it may become necessary after two or three days if larger amounts are passed through.

EXPERIMENTAL

1. *Determination of the Numbers of Organisms Contained in Freshly Prepared and Chemically Suitable Effluent at Intervals during Its Production.*—One-milliliter amounts of effluent samples collected from 2 different units at random times during a period of forty-two days were plated for bacterial count. All agar plates were incubated for seventy-two hours at 37°. The results (Table I) showed a considerable degree of variation in the bacterial content of the various samples and indicated that the numbers of organisms in the effluent bore a direct relationship to the time elapsing between successive regenerations.

TABLE I.—RELATIONSHIP BETWEEN BACTERIAL CONTENT OF CHEMICALLY SUITABLE DEIONIZED WATER AND FREQUENCY OF REGENERATIONS

Days After Previous Regeneration	Bacteria/Ml in Influent	Bacteria/Ml in Effluent
	Unit I	
7	0	1
1	0	0
2	1	32
1	4	0
3	0	0
7	0	150
2	4	0
8	3	5
	Unit II	
1	5	1
8	14	16
16	0	520
22	0	110
34	1	700
42	2	125

2. **Relationship between the Chemical Quality of Deionized Water and Its Corresponding Bacterial Content.**—Immediately following the process of regeneration of the resins and prior to the issuance of chemically suitable water as indicated by the system of colored lights, 100-ml. samples of water were collected aseptically at frequent intervals and plated for count. Additional samples were also taken during periods ranging from twelve hours to twenty days after regeneration. For this study, the same two deionizing units as used in the preceding experiment were available, one of which (Unit I), being used almost continuously, was regularly regenerated at intervals of no greater than three or four days, while the other (Unit II), operated infrequently, was regenerated approximately every twenty days. From the results obtained (Tables II and III) it becomes apparent that the bacterial content of the effluent water bears no relationship to its chemical quality.

A very important difference between the two units is readily apparent. In Unit I, which is regenerated every three or four days, there is but little growth of bacteria since the product is practically sterile in spite of a count of 60 bacteria per ml. in the raw water. On the other hand, in Unit II, which requires infrequent regenerations, there is considerable growth and release of bacteria. Actually, this unit produces water containing more than 100 times as many organisms per ml. as are contained in the pretreated water, and this in spite of the fact that the product is chemically pure.

3. **Types of Bacteria Found in Deionized Water.**—Since relatively large numbers of bacteria were encountered in the water issuing from the demineralizing units, a survey was made to determine the types present. The predominating organisms found in freshly prepared deionized water were pseudomonas types normally present in fresh water. The remaining forms were Gram-negative, nonmotile, or motile rods. Spore-forming bacilli were not generally observed. From these findings it would seem that the synthetic resins do not alter the flora of the water passing through them.

DISCUSSION

The data presented in this report clearly indicate that deionized water differs from distilled water with respect to its bacterial content. The presence or absence of bacteria depends upon the frequency of use of the units. After fresh regeneration, the resins are capable of removing the small numbers of bacilli contained in the raw water during the subsequent first few hours and the product is sterile or very nearly so. However, with infrequent regenerations, the resin beds represent a culture medium for retained bacteria which are flushed out with the effluent when the unit is placed into operation. This is readily apparent when it is shown that the raw water entering the unit contains from 40 to 90 organisms per ml. while the resulting deionized water, although chemically acceptable, contains upward of 6000 to 12,000 organisms per ml. Indeed, as has been found in our studies, subsequent storage of this product containing, in addition to bacilli, organic matter not removed by the resins may result in a bacterial content exceeding a million organisms per ml. within twenty-four hours after preparation. The implications with respect to the introduction of pyrogens as a result of excessive bacterial growth are worthy of serious consideration. On the other hand, it is possible to produce demineralized water relatively free of microorganisms provided that the units are used under conditions similar to those employed with Unit I; that is, daily use and regeneration of the resins every third or fourth day.

SUMMARY

A bacteriological study has been made of demineralizing units employing synthetic cation and anion exchange resins for the production of water with low mineral content. It has been established that there exists a wide variation

TABLE II.—CORRELATION BETWEEN BACTERIAL NUMBERS AND CHEMICAL PURITY OF DEIONIZED WATER—UNIT I

Time of Sampling, Min.	Freshly Regenerated		12 Hours after Regeneration		24 Hours of Regeneration	
	Bacteria Per Ml.	Chemical Quality[a]	Bacteria Per Ml.	Chemical Quality	Bacteria Per Ml.	Chemical Quality
Start	0	Poor	0	Poor	210	Poor
5	1	Poor	0	Good	0	Poor
10	0	Poor	0	Good	0	Good
15	0	Poor	0	Good	2	Good
20	0	Poor	0	Good	0	Good
30	5	Poor	2	Good	4	Good
40	0	Poor	0	Good	0	Good
50	0	Poor	1	Good	0	Good
60	1	Poor	0	Good	0	Good
70	0	Good	0	Good	2	Good
80	0	Good	0	Good	0	Good
90	0	Good	0	Good	1	Good
120	0	Good	0[b]	Good	0[c]	Good
Raw water supply	60		40		34	

[a] As indicated by the electrical conductivity of the water.
[b] Total output: 150 gallons.
[c] Total output: 300 gallons.

TABLE III.—CORRELATION BETWEEN BACTERIAL NUMBERS AND CHEMICAL PURITY OF DEIONIZED WATER—UNIT II

Time of Sampling, Min.	Freshly Regenerated		144 Hours after Regeneration		20 Days after Regeneration	
	Bacteria Per Ml.	Chemical Purity	Bacteria Per Ml.	Chemical Purity	Bacteria Per Ml.	Chemical Purity
Start	0	Poor	6500	Poor	12,000	Poor
5	0	Poor	5220	Poor	3,600	Poor
10	1	Poor	800	Poor	4,900	Poor
15	1	Poor	540	Poor	5,100	Poor
20	2	Poor	300	Good	6,100	Good
30	10	Poor	450	Good	2,200	Good
40	21	Poor	660	Good	890[b]	Good
50	27	Poor	1000	Good	360	Poor
60	17	Good	1440	Good	390	Poor
70	20	Good	1220	Good	550	Poor
80	22	Good	700	Good	1,020	Poor
90	26	Good	150[a]	Good	1,000	Poor
Raw water supply	300		40		90	

[a] Total output: 175 gallons.
[b] Total output: 645 gallons.

in bacterial content in deionized water which is conditioned by the manner in which these units are operated. An almost sterile product may be produced provided the deionizing units are used daily and the resins regenerated every three or four days. If, on the other hand, the resins are regenerated at infrequent periods, that is, several days or weeks apart, the bacterial content of the effluent, notwithstanding its chemical purity, may be excessive, being considerably greater than the numbers of organisms in the raw water entering the unit. The storage of such water gives rise to exceedingly large numbers of bacteria within twenty-four hours after collection.

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The Solubility of Theophylline in Aqueous Amine Solutions*

By E. E. LEUALLEN† and ARTHUR OSOL‡

A study has been made of the solubility of theophylline in aqueous solutions of certain amines. The molar solubility of theophylline has been plotted against molar concentrations of monomethylamine, dimethylamine, trimethylamine, monoethylamine, diethylamine, mono-*n*-propylamine, di-*n*-propylamine, mono-*n*-butylamine, and di-*n*-butylamine. The solubilizing effect decreases with increasing length of the carbon chain; thus monomethylamine has an effect greater than that of any other amine investigated. Monomethylamine has greater solubilizing effect than does dimethylamine, which in turn is more effective than trimethylamine. The solubility increases with increasing concentration of primary amine until a point of maximum solubility is reached, thereafter solubility decreases with increasing amine concentration. The maximum solubility increases with increasing length of the carbon chain.

MANY substances have been shown to increase the water solubility of theophylline, with the great majority of these substances being amines. In some instances double compounds have been isolated and a few of these have become important therapeutic commodities. In 1907 a German patent (1) was issued for the preparation of such a compound by reaction of theophylline and piperazine and subsequent evaporation *in vacuo* to obtain a solid product. Additions to this patent covered the reaction of barium theophylline and piperazine sulfate (2), the reaction of theophylline and such amines as ethylenediamine, hexamethylenediamine and lysidine (3), and the fact that these amines produced soluble double compounds in less than molar ratios (3,4). The first United States patent (5) for this type of product was issued in 1909 to the same organization. This patent discloses that theophylline forms water soluble compounds with primary and secondary diamines and indicates the use of ethylenediamine, pentamethylenediamine, and hexamethylenediamine.

Among other substances which have been shown to be solubilizing agents for theophylline are monoethanolamine (6), diethanolamine (7), triethanolamine (6), chlorophylline (8), monoamino polyhydric alcohols such as glucamine, methyl glucamine, ethyl glucamine, dimethyl glucamine and ethanol glucamine (9, 10), mono-, di- and tri-isopropanolamine (11), β-phenylisopropylamine and its derivatives (12) and 2-diethylaminoethanol (13). Greenbaum has stated (14) that the compounds formed from theophylline and either morpholine, propylenediamine, ethylamine, piperidine or triethylenetriamine, in monomolecular and polymolecular proportions, were not sufficiently soluble in water to be therapeutically useful. Complex compounds of theophylline and salts of *ortho*- or *meta*-hydroxybenzoic acid have been described in several patents (15–19). Compounds or molecular associations have also been reported to be formed with dehydrocholic acid (20), phenobarbital (21–23), 3-chloro-1,2-dihydroxypropane (24), and papaverine (25). A product identified as theophylline aminoisobutanol has been studied experimentally and clinically (26–28).

In the present study a determination has been made of the solubility of theophylline in aqueous solutions of the following aliphatic amines: monomethylamine, dimethylamine, trimethylamine, monoethylamine, diethylamine, mono-*n*-propylamine, di-*n*-propylamine, mono-*n*-butylamine and di-*n*-butylamine. Several concentrations of each amine have been prepared in order to observe the effect of increasing concentration on solubilizing ability. Findings are reported in Tables I to IX.

EXPERIMENTAL

1. **Materials.**—The theophylline used in this study was a U. S. P. product, recrystallized from water and dried at 100°. The amines were commercial grade supplied by Eastman Kodak in aqueous solution. Dilutions were made with freshly distilled water.

2. **Method.**—In each determination an excess of theophylline was added to the amine dilution contained in a 200-cc. hard glass bottle and a paraffined cork inserted and tied in place. The bottles were rotated in a constant temperature bath containing water at 25° ± 0.02° for not less than forty-eight hours. The excess solute was permitted to

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Presented to the Scientific Section, A. PH. A., San Francisco meeting, 1948.
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TABLE I.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF MONOMETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.5364	0.8399
0.7903	1.200
1.028	1.520
1.244	1.783
1.696	2.293
1.802	2.409
1.809	2.376
1.991	1.848
2.106	1.780
2.691	1.594
3.538	1.414
3.779	1.376

TABLE II.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF DIMETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.3081	0.5140
0.6355	0.9850
1.008	1.470
1.238	1.743
1.695	2.235
1.765	2.302
2.356	2.868
2.955	3.259

TABLE III.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF TRIMETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.2525	0.3905
0.5164	0.7367
0.8411	1.093
1.078	1.376
1.532	1.743
1.930	2.060
2.418	2.366

TABLE IV.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF MONOETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.4982	0.7818
1.021	1.480
1.750	2.305
1.812	2.357
2.327	2.869
2.406	2.677
2.437	2.666
2.806	2.397

TABLE V.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF DIETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.4723	0.7462
1.066	1.503
1.335	1.813
1.379	1.864
2.170	2.558
2.669	2.916

TABLE VI.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF MONO-*n*-PROPYLAMINE

Molarity of Amine	Molarity of Theophylline
0.7020	1.080
1.115	1.620
2.458	2.913
2.605	2.895
2.918	2.730
3.321	2.553
3.687	2.446

TABLE VII.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF DI-*n*-PROPYLAMINE

Molarity of Amine	Molarity of Theophylline
0.4398	0.6965
0.7925	1.163
1.281	1.680
1.356	1.735
1.409	1.804
1.556	1.945
2.207	2.399
2.243	2.436

TABLE VIII.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF MONO-*n*-BUTYLAMINE

Molarity of Amine	Molarity of Theophylline
0.6063	0.9379
1.154	1.629
2.481	2.863
2.499	2.886
2.721	3.071
2.811	3.087
2.886	3.030
4.132	2.605
4.491	2.489

TABLE IX.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF DI-*n*-BUTYLAMINE

Molarity of Amine	Molarity of Theophylline
0.3396	0.5580
0.5379	0.8190
0.6707	0.9954
1.238	1.636
1.822	2.110
1.940	2.196
2.125	2.278
2.212	2.320
2.490	2.460
2.648	2.496
2.694	2.502

settle and samples of the supernatant saturated solution were withdrawn by means of a dry 10-cc. calibrated pipette, to the lower orifice of which was fastened by means of rubber tubing a 5-cm. length of glass tubing having a drawn end to permit easy attachment and glass-to-glass contact; cotton placed in this tube served as a filter. In each solution, the first portion of fluid drawn through the cotton was discarded. Samples of appropriate size were placed in each of two weighing bottles and in a pycnometer. One sample was used for the determination of amine content, one for theophylline content, and that in the pycnometer for the determination of specific gravity.

Amine content was determined by titration with 0.1 N H_2SO_4 using methyl red indicator solution. Theophylline content was determined by the procedure described in the U. S. P. XII under Theophylline Ethylenediamine. The specific gravity was used to convert sample weights to sample volumes in order to calculate molarities.

DISCUSSION

Plotting amine concentrations, in molarity, against the solubility of theophylline, also expressed as molarity, the curves shown in Figs. 1 to 3 were obtained. In Fig. 1, a comparison of the solubilizing effect of four primary amines indicates that for any given molarity below the point of maximum solubility, the solubilizing effect decreases with increase in length of the carbon chain. It is also evident, however, that the maximum solubility increases with increase in length of the carbon chain. Inasmuch as no simple or constant ratio of theophylline to amine concentration was found to hold at any of the several points of maximum solubility, no conclusions have been drawn concerning compound formation.

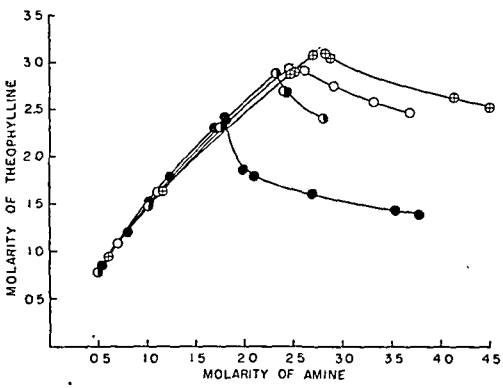

Fig. 1.—Primary amines effect on solubility of theophylline. ●, methylamine; ◑, ethylamine; O, n-propylamine; ⊕, n-butylamine.

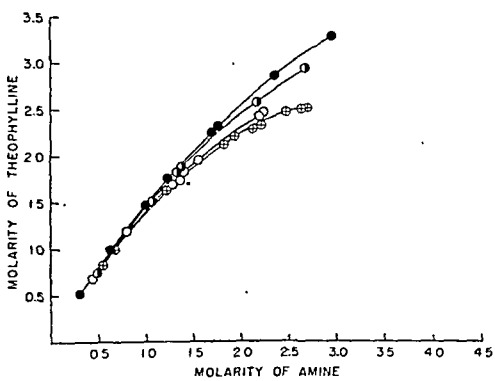

Fig. 2.—Secondary amines effect on solubility of theophylline. ●, dimethylamine; ◑, diethylamine; O, di-n-propylamine; ⊕, di-n-butylamine.

In Fig. 2, which depicts the behavior of dialkylamines, it is again evident that at a given amine concentration, the solubilizing effect decreases with increasing length of the carbon chain. With these secondary amines points of maximum solubility were not reached although preliminary observations have indicated that the amine concentrations here employed for di-n-propylamine and di-n-butylamine are probably approaching the point of maximum solubilization.

In Fig. 3, a comparison of the effect of the methyl amines indicates that for any given concentration of amine below the point of maximum solubility of theophylline, the monomethylamine has the greatest solubilizing action but that the maximum solubility obtained with monomethylamine is not so great as that obtainable with dimethylamine or trimethylamine.

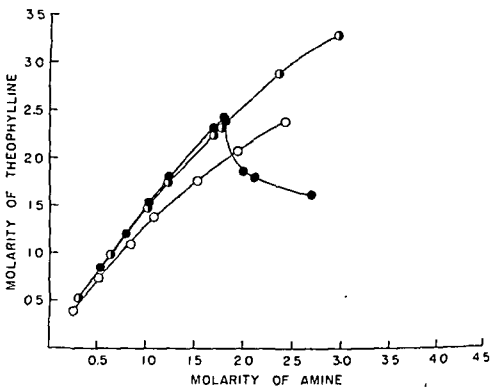

Fig. 3.—The methylamines effect on solubility of theophylline. ●, methylamine; ◑, dimethylamine; O, trimethylamine.

It may also be seen from Figs. 1 and 2 that in all instances a monoalkylamine has a greater solubilizing effect on theophylline than does the corresponding dialkylamine in the region below that of the maximum solubility of theophylline.

SUMMARY

1. The solubility of theophylline in certain amine-water systems has been determined.

2. By plotting amine molarity against theophylline molarity it has been shown that, as a general rule, in dealing with lower concentrations of amines, the solubility of theophylline increases with increasing amine concentration.

3. In the case of primary amines, the solubility was found to increase with increasing concentration of amine until a point of maximum solubility was reached, after which the solubility decreased with increasing amine concentration. This maximum solubility increased with increasing length of the carbon chain.

4. The molar solubilizing effect of monomethylamine is greater than that of dimethylamine, which, in turn, is more effective than trimethylamine.

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A Bactericidal Spectrum of Some Common Organisms*

By MORRIS OSTROLENK and C. M. BREWER

The reactions of fourteen selected microorganisms to critical germicidal concentrations of twelve chemical compounds are presented. The use of a greater variety of commonly occurring bacteria as an adjunct to conventional germicidal testing methods is briefly discussed.

THE TERM "germicide" is defined as a substance or agent that kills germs. Unless specifically qualified, limits and restrictions on the percentage or types of germs killed do not find a place in this definition; with the exception that it is customary to exclude bacterial spores from the requirements of effective germicidal action. In spite of the fact that interest in germicidal activity extends into the past as far back as our concern with bacteriology itself, and that the difference in susceptibility to germicides was early recognized, with the exception of two microorganisms, criteria of representative resistance of different types of bacteria for germicidal testing purposes have not yet been established. Only from *Micrococcus pyogenes* var. *aureus* and *Salmonella typhosa* have strains been deliberately selected to represent standard resistance of their species to germicidal action. A strain of *M. aureus* was designated by Reddish (3) following the initiatory work of Shippen, and, earlier, by Anderson and McClintic (1) in the case of *S. typhosa*.

The fallacy of placing reliance on the reaction of a single strain of test organism to bactericides as a measure of germicidal potentiality has been recognized since the earliest studies when various bacterial species were used. Among the many who have advocated or utilized different species of microorganisms in more recent studies of germicidal activity, Philbrick (2), Reddish (4), Tilley (5), Walters (6), Zoller and Eaton (7) may be cited. Scientific literature is noticeably lacking in germicidal data on the use of a number of strains of different species of test organisms that have been standardized against one or more chemically defined compounds. For entirely too long, tests limited to the reactions of *S. typhosa* (Hopkins) and *M. aureus* toward antibacterial preparations under rigid sets of conditions have been widely exploited and accepted as a means of estimating antiseptic and disinfectant efficacy. Standardization of tests offering comparison under arbitrary conditions has generally been favored over measurement by practical accomplishment. Neither aspect can properly be neglected. An ideal standardized method that can compare antibacterial potentialities and at the same time serve as an indication of efficacy has long been sought. Such a test is yet to be realized. The use of a Gram-negative organism (*S. typhosa*) relatively susceptible to germicidal substances, together with a comparatively resistant Gram-

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positive strain of microorganism (*M. aureus*), may afford a reasonable amount of justification for estimating the limits of effective concentrations of germicides to be used as disinfectants on inanimate surfaces. The established precedent of recommending a disinfectant for practical application at concentrations equivalent to twenty times its phenol coefficient value, which is theoretically comparable to the use of 5 per cent phenol, provides, in general, an acceptable margin of safety. When, however, germicides are used as antiseptics on living mammalian tissue where toxicity of the agent may be of greater importance than germicidal potency, no such factor of bactericidal safety can feasibly be employed.

In order to obtain more explicit information on germicidal testing, strains from a number of different microorganisms were selected and tested against several well-recognized chemical compounds. The significance of the work lies in the uniform selection and testing of various organisms against a variety of antibacterial chemical agents. In this respect only is the presented material unique. No original concept motivated this accumulation of data. No attempt to introduce innovations in currently used methods or recommendation of a particular procedure is intended. These data were obtained solely for our own information and are offered only as of possible interest to others engaged in germicidal testing. The limited results omit many important groups of both organisms and chemical compounds, and thus belie the title. However, the demonstration of a complete spectrum of germicidal values is obviously beyond the scope of a single piece of work.

EXPERIMENTAL

Methods and Materials.—Organisms were taken from nutrient agar slants and carried continuously in an unadjusted broth containing only 2% trypticase. Its *p*H was 6.6–6.8 after sterilization. All organisms were transferred daily, using one 4-mm. loopful of inoculum, and grown at 37°. For subculture after exposure to germicidal agents, 15-ml. amounts of a fluid thioglycollate medium were used. Selection of 14 representative test organisms from nine genera was based on reaction to phenol at 20°. Test organisms were selected as representing the more resistant strains of their respective genera and on the basis of uniformity of reaction on repeated tests against phenol. Each test organism, except *S. typhosa* (Hopkins), *M. aureus* (209), and *Serratia marcescens*, was selected from groups composed of from four to 15 strains. The selected test organisms were grown and transferred as before. The number of viable cells per ml. was estimated for each test culture. These counts were made by seeding serial dilutions in nutrient agar. Counts were made on four separate occasions and each was made in triplicate. The estimated number of viable cells for each organism remained quite uniform throughout the tests. The names, designations and viable counts per ml. are given in Table I. The chemical compounds used in the tests are given in Table I-A.

TABLE I.—TEST ORGANISMS

Organism	Designation	Bacterial Count per Ml.[a]
Micrococcus pyogenes var. *aureus*	*S.a*	725,000,000
Micrococcus pyogenes var. *albus*	*S-2*	650,000,000
Micrococcus pyogenes sp.	*S-1*	400,000,000
Micrococcus pyogenes (human throat)	*E-2*	290,000,000
Streptococcus (viridans type)	*S-3*	41,500,000
Streptococcus fecalis	*St. fec.*	560,000,000
Corynebacterium hofmanni	*C. hof.*	147,500,000
Corynebacterium ovis	*C. ovi.*	300,000,000
Eberthella typhosa	*E. ty.*	990,000,000
Pseudomonas aeruginosa	*Ps. aer.*	3,600,000,000
Escherichia coli	*E. col.*	1,500,000,000
Aerobacter aerogenes	*A. aer.*	1,300,000,000
Serratia marcescens	*S. mar.*	1,500,000,000
Proteus vulgaris	*P. vul.*	750,000,000

[a] Trypticase broth (24 hours at 37°).

TABLE I-A.—COMPOUNDS USED FOR TESTING RELATIVE RESISTANCE AND SUSCEPTIBILITY OF TEST ORGANISMS[a]

Compound	Abbreviation
Alcohol (ethyl)	Et alc
Alcohol (Isopropyl)	I P alc
Hydrochloric acid	HCl
Hydrogen peroxide	H_2O_2
Iodine (aqueous sol.)	I_2
Saponated Cresol solution	Cresol
Mercuric chloride	HgCl
Carbolic acid	Phenol
Phenyl mercuric acetate	PMA
Phenyl mercuric nitrate	PMN
Silver nitrate	$AgNO_3$
Sodium hydroxide	NaOH

[a] Dilutions in the tables represent absolute concentrations except under the caption "Cresol," where concentrations of the U. S. P. solution are represented.

The testing technique employed was the phenol coefficient type of method (drop method). The procedure differed from that described in the A. O. A. C. Methods of Analysis[1] only in the media used, and the four-day period of subculture incubation.

Reactions of the organisms to the various chemicals were tested at 20° and 37°. The results are arranged in tables, one for each of the 12 chemical reagents, and show survival or susceptibility of each organism after five, ten, and fifteen minutes' exposure over the range of critical dilutions of the germicides. The recorded results shown as single tests actually represent compilations of all tests for any one organism against any one compound, with the occasional exception of an obviously aberrant result. The individual trial tests varied from 4 to 27, as indicated by the figure at the foot of each column.

[1] The same procedure originally described in U. S. Department of Agriculture Circular No. 198.

With the exception of phenol, which was utilized frequently to check the resistance of the test organisms, relatively large numbers of trial tests were performed when inconsistent results were obtained. Growth in the subculture, indicating survival of the exposed organism is designated "+" when this result was obtained in no fewer than two-thirds of the trial tests. Similarly, when no growth occurred in the subcultures in at least two-thirds of the tests the result is designated "0." When inconsistencies resulted at any one concentration time period so that positive and negative results occurred about equally, actually between one-third and two-thirds of the trial tests, the result is designated as "0/+."

In each table, the reactions of the individual test organisms have been arranged in a descending order of resistance when tested at 20°. The summarized results are given in Tables II–XIII.

RESULTS

From the compiled data offered in the tables all the information accumulated in this study can be obtained. Several observations, however, appear to warrant special attention. Most striking is the interchange of relative susceptibility of the organisms to different reagents. Except in the case of the mercurial compounds, where sufficiently concentrated solutions to effect germicidal action could not be obtained for many of the organisms, the relationship of resistance was not the same toward any two chemical reagents. Considering the Gram-positive and Gram-negative groups independently, not only was every organism found at one or the other extreme on the scale of comparative resistance in its reactions to different compounds, but in several instances the organism most susceptible to one compound was the most resistant to another. As examples: The staphylococcus designated "*S-2*" resisted a higher concentration of cresol than any other organism but was the most susceptible to the action of iodine. In the Gram-negative group *P. vulgaris* exhibits the most resistance to hydrochloric acid and *Ps. aeruginosa* the least; whereas, tested against iodine the reverse situation is encountered.

From the tables can be obtained the range of germicidal dilutions, selective or nonselective action, the comparative reactions of the Gram-positive group and Gram-negative group, and the differences in temperature reactions, elicited by the different compounds.

It can be observed from the charts that some germicidal compounds have a marked selective action against the Gram-negative group of organisms. This is the case with sodium hydroxide and, within a very narrow range, the two alcohols. The converse was found when silver nitrate was used as a testing

TABLE II.—PHENOL

		Test Organisms							
		Gram-positive							
	Diln.	*St. fec.*	*S-2*	*S-a*	*S-1*	*C. hof.*	*S-3*	*C. ovi.*	*E-2*
20°	1:50	0 0 0	...	0 0 0	...	0 0 0	...	...	...
	1:60	++0/+	+0 0	+0 0	0/+0 0	0/+0 0	0/+0 0	0 0 0 0	0 0 0
	1:70	+++	+++	++0/+	+ ++	+ +0/+	+ 0/+0	+0 0	+0 0
	1:80	...	...	+++	...	+ ++	+ + 0/+	++ +	++0/+
	1:90	...	...	...	...	...	+ + +	...	+++
No. of tests		14	6	27	6	6	9	6	9
37°	1:70	0 0 0	0 0 0	...	...	...	...	...	...
	1:80	+0/+0	+0 0	0 0 0	0 0 0	...	...	...	...
	1:90	++ +	++0/+	+0/+0	+0 0	0 0 0	...	0/+0 0	0 0 0
	1:100	...	+++	++ +	+++	+0 0	0/+0 0	+0/+ 0	+0 0
	1:110	...	...	...	...	++0/+	+ +0	+ + +	++0/+
	1:120	...	...	...	...	+++	+ ++	...	+++
No. of tests		9	6	19	6	5	10	5	11

		Gram-negative					
	Diln.	*E. col.*	*S. mar.*	*A. aer.*	*Ps. aer.*	*E. typhosa*	*P. vul.*
20°	1:60	0/+0 0	0 0 0	0 0 0	0 0 0	...	...
	1:70	+ ++	+0 0	0/+0 0	0/+0 0	0 0 (	0 0 0
	1:80	...	+++	+ + 0	+ +0/+	+0/+ 0	+0 0
	1:90	...	...	+ ++	+ ++	++ +	+++
No. of tests		16	5	5	11	18	5
37°	1:80	0 0 0	...	...	...	...	...
	1:90	+0/+0	...	...	...	...	...
	1:100	++ 0/+	0 0 0	...	...	...	...
	1:110	++ +	++0	0 0 0	0/+0 0	...	...
	1:120	...	++0/+	0/+0 0	+ +0	...	0 0 0
	1:130	...	+++	+ 0 0	+ +0/+	0 0 0	0/+0 0
	1:140	...	...	+ +0/+	+ ++	+0 0	+ 0 0
	1:150	...	...	+ ++	...	++ 0	+ +0
	1:160	...	...	...	...	++ 0/+	+ ++
	1:170	...	...	...	...	++ +	...
No of tests		10	6	5	9	11	5

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE III.—PHENYLMERCURIC NITRATE

		Test Organisms							
		Gram-positive							
	Diln.	*S-a*	*St. fec.*	*S-3*	*S-1*	*S-2*	*E-2*	*C. hof.*	*C. ovi.*
20°	1:1350	+++	+++	+++	+++	+++	+++	+++	++0/+
	1:2700	...	...	...	...	...	...	...	+++
No. of tests		8	8	7	6	6	7	6	8
37°	1:1350	+++	+++	+++	+++	+++	0 0 0	...	...
	1:2700	...	..	...	..	.	+++	0 0 0	0 0 0
	1:6750	...	...	...	...	...	..	+++	+++
No. of tests		8	8	7	6	6	7	6	8

		Gram-negative					
	Diln.	*E. col.*	*P. vul.*	*S. mar.*	*Ps. aer.*	*E. typhosa*	*A. aer.*
20°	1:1350	+++	+++	+++	+++	+++	+++
No. of tests		4	5	5	5	5	4
37°	1:1350	++0/+	+0/+0	+0/+0	+0 0	+0 0	0/+0 0
	1:2700	+++	++ +	++ 0/+	++0	+0/+0	+ 0 0
	1:6750	. .	. .	++ +	+++	+0/+0/+	+ +0/+
	1:13,500	...	..	...	...	++ +	+ ++
No. of tests		4	5	5	5	5	4

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE IV.—PHENYLMERCURIC ACETATE

		Test Organisms							
		Gram-positive							
	Diln.	*S-1*	*St. fec.*	*S-3*	*S-a*	*E-2*	*S-2*	*C. hof.*	*C. ovi.*
20°	1:350	+++	++ +	++ +	++ 0/+	0/+ 0 0	0 0 0	0 0 0 0	...
	1:700	...	..	.	++ +	+ 0/+0	+0/+ 0	0/+0 0	0 0 0
	1:1750	...	...	...	...	+ + +	++ +	+ + 0/+0	+0/+0
	1:3500	...	...	...	....	...	...	+ + +	+0/+0/+
	1:5250	...	. .	...	...	...	...	...	++ +
No. of tests		7	6	6	6	8	5	5	6
37°	1:350	+++	+0/+0/+	0 0 0	0 0 0	...	...	...	...
	1:700	..	++ +	0/+0 0	0/+ 0/+ 0	0 0 0	0 0 0	0	...
	1:1750	...	. .	+ 0/+0	+ + 0/+	0/+ 0 0	+0 0 0	0 0 0	...
	1·3500	...	...	+ + 0/+	+ + +	+ 0 0	++ + +	+ 0/+0	0 0 0
	1:5250	...	...	+ + +	...	+ 0 0	...	+ + 0/+++	+
	1:7000	...	...	.	...	+ 0 0	...	+ + +	...
	1:8750	...	. .	.	...	+ + 0	...	...	...
	1:10,500	...	...	. .	. .	+ + +	. .	...	...
No. of tests		4	5	4	5	5	4	5	4

		Gram-negative					
	Diln.	*E. col.*	*A. aer.*	*Ps. aer.*	*P. vul.*	*E. typhosa*	*S. mar.*
20°	1:350	0/+ 0 0	0 0 0	0 0 0	0 0 0	...	...
	1:700	+ 0 0	+0/+0	0/+0 0	+0 0	0 0 0	0 0 0
	1:1750	+ ++	++ +	+ +0/+	++0/+	++0/+	+0 0
	1:3500	...	...	+ ++	+++	+++	+++
No. of tests		5	5	4	6	4	4
37°	1:1750	0 0 0	. .	0 0 0	0 0 0	...	...
	1:3500	+ 0/+0	0 0 0	+0/+0/+	+0 0	...	0 0 0
	1:5250	+ + 0	..	...	...	...	...
	1:7000	+ + +	+0 0	++ 0/+	++0	0 0 0	+0 0
	1:10,500	...	+0 0	++ +	+++	0/+0 0	++0
	1:14,000	..	+0/+0	...	..	+ 0 0	+++
	1:17,500	..	+0/+0	...	..	+ 0 0	...
	1:21,000	...	++ 0/+	...	...	+ 0/+0	...
	1:24,500	...	++ +	...	...	...	...
	1:28,000	...	...	...	...	+ + 0	...
	1:35,000	...	...	...	...	+ + +	...
No. of tests		7	6	7	5	6	5

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE V.—MERCURIC CHLORIDE

		Test Organisms							
		Gram-positive							
	Diln.	*S-a*	*St. fec.*	*S-1*	*E-2*	*S-3*	*S-2*	*C. hof.*	*C. ovi.*
20°	1:20	+++	+ + +	+++	++ 0/+	+0/+ 0/+	0/+0/+0	...	...
	1:30	...	..	..	++ +	++ +	+ 0/+0/+	...	...
	1:40	...	..	..	.	...	+ 0/+0/+	0 0 0	0 0 0
	1:50	...	...	..	.	...	+ + +	0/+0/+0/+	0/+0 0
	1:60	..	...	.	.	...	...	+ + +	+ 0/+0/+
	1:70	...	...	...	..	...	...	...	+ + 0/+
	1:80	...	...	...	...	...	...	...	+ + +
No. of tests		5	9	4	5	5	4	4	4
37°	1:20	0/+0 0	0/+0 0	++0/+	..	..	...	...	...
	1:30	0/+0 0	+ 0/+0/+	+++	0 0 0	...	...	...	...
	1:40	+ +0	+ + +	..	+0 0	0 0 0	...	...	...
	1:50	+ +0	..	.	+0 0	0/+0 0	...	...	..
	1:60	+ +0/+	...	..	+0 0	0/+0 0	...	...	...
	1:70	+ ++	...	...	+0/+0	+ 0 0	0 0 0	..	00 0
	1:80	...	.	...	+0/+0	+ 0/+0	0/+0 0	0 0 0	0/+0 0
	1:90	...	...	...	+0/+0	+ + 0	0/+0 0	0/+0 0	+ 0 0
	1:100	...	..	...	+0/+0	+ + 0	0/+0 0	0/+0 0	+ 0 0
	1:200	...	...	...	+0/+0	+ + 0/+	+ 0/+0	+ + +	+ + 0
	1:300	...	...	...	++ 0	+ + +	+ 0/+0	..	+ + 0/+
	1:400	...	...	...	++ 0/+	...	+ 0/+0	...	+ + +
	1:500	...	...	...	++ +	...	+ + 0	...	...
	1:600	...	...	...	...	...	+ + +	...	...
No. of tests		7	7	7	7	6	6	6	6

		Gram-negative					
	Diln.	*E. col.*	*S. mar.*	*A. aer.*	*E. typhosa*	*P. vul.*	*Ps. aer.*
20°	1:20	++ 0/+	+ +0/+	+ + 0/+	0/+0/+0	0/+0 0	...
	1:30	++ +	+ +0/+	+ + 0/+	+ + 0/+	+ 0/+0	..
	1:40	...	+ ++	+ ++	+ + +	+ + 0	.
	1:50	...	...	...	...	+ + 0/+	...
	1:60	...	...	...	...	+ + +	...
	1:80	...	...	...	...	...	0 0 0
	1:90	...	...	...	....	...	0/+0 0
	1:100	...	...	...	...	...	0/+0 0
	1:200	...	...	...	...	...	+ 0/+0/+
	1:300	...	...	...	...	...	+ + 0/+
	1:400	...	...	...	...	...	+ + +
No. of tests		5	5	5	5	4	5
37°	1:20	0 0 0	0/+0 0	...	...	...	...
	1:30	0/+0 0	...	...	...	...	...
	1:40	+ 0 0	0/+0 0	...	...	...	...
	1:50	+ +0	...	0 0 0	0 0 0	...	...
	1:60	+ +0/+	+ 0 0	0/+0 0	0/+0 0	...	...
	1:70	+ ++	+ 0 0	0/+0 0	0/+0 0	0 0 0	...
	1:80	...	+ 0 0	0/+0/+0	0/+0 0	0/+0 0	
	1:90	...	+ 0 0	+ 0/+0	+ 0 0	0/+0 0	...
	1:100	...	+ +0	+ 0/+0	+ 0 0	+ 0 0	...
	1:200	...	+ ++	+ + +	+ + 0	+ 0 0	.
	1:300	...	...	...	+ + 0	+ + 0	0 0 0
	1:400	...	...	...	+ + 0/+	+ + +	0/+0 0
	1:500	...	...	...	+ + +	...	0/+0 0
	1:600	...	...	...	...	...	0/+0 0
	1:700	...	...	...	...	...	0/+0/+0
	1:800	...	...	...	...	...	+ 0/+0
	1:900	...	...	...	...	...	+ + +
No. of tests		5	5	7	5	7	6

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE VI.—ALCOHOL

		Test Organisms — Gram-positive							
	Per Cent	*S-1*	*St. fec.*	*E-2*	*S-2*	*S-a*	*C. ovi.*	*C. hof.*	*S-3*
20°	54	0 0 0	0 0 0	...	...	...	...	...	...
	52	+ 0/+0	0/+0 0	0 0 0	0 0 0	0 0 0	...	...	...
	50	+ + 0	+ 0 0	+ 0 0	0/+0 0	0/+0 0	0 0 0	0 0 0	...
	48	+ + +	+ ++	+ 0/+0	+ 0 0	+ 0 0	+ 0 0	0/+0 0	0 0 0
	46	...	...	+ + +	+ + +	+ + 0/+	+++	+ ++	++0/+
	44	...	...	...	...	+ + +	...	...	+++
No. of tests		8	9	10	5	13	5	6	12
37°	38	0 0 0	...	...	0 0 0	...	...	...	...
	36	0/+0 0	0 0 0	...	0/+0 0	...	...	...	...
	34	+ + 0/+	+ 0 0	0 0 0	+ 0/+0	0 0 0	0 0 0	...	...
	32	+ + +	+ +0	0/+0 0	+ 0/+0	0/+0 0	0/+0 0	0 0 0	...
	30	...	+ ++	+ + 0	+ + +	+ 0/+0	+ 0 0	+ 0 0	...
	28	...	...	+ + 0/+	...	+ + 0/+	+ +0/+	+ 0/+0	0 0 0
	26	...	...	+ + +	...	+ + +	+ ++	+ + +	+0 0
	24	...	..	...	...	...	...	...	+++
No. of tests		8	9	9	8	12	5	5	10

		Gram-negative					
	Per Cent	*E. col.*	*P. vul.*	*S. mar.*	*A. aer.*	*Ps. aer.*	*E. typhosa*
20°	46	0 0 0	0 0 0	0 0 0	...	...	...
	44	0/+0 0	+ 0 0	0/+0 0	0 0 0	0 0 0	...
	42	+ +0/+	+ 0/+0	+ 0/+0	0/+0 0	0/+0 0	...
	40	+ ++	+ + +	+ + +	+ 0/+0	+ 0 0	0 0 0
	38	...	...	...	+ + 0/+	+ 0/+0	0/+0 0
	36	...	...	...	+ + +	+ + +	+ +0/+
	34	...	...	...	...	...	+ ++
No. of tests		11	7	6	11	13	11
37°	30	0 0 0	...	...	...	...	...
	28	0/+0 0	0 0 0	0 0 0	...	...	...
	26	+ +0/+	0/+0 0	0/+0 0	0 0 0	0 0 0	...
	24	+ ++	+ 0 0	+ 0/+0	0/+0 0	0/+0 0	0 0 0
	22	...	+ + 0	+ + 0/+	+ 0 0	+ 0 0	+ 0 0
	20	...	+ + +	+ + +	+ + 0	+ + 0/+	+ 0/+0
	18	...	...	...	+ + +	+ + +	+ + 0/+
	16	...	...	...	...	...	+ ++
No. of tests		10	5	5	10	12	10

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE VII.—ISOPROPYL ALCOHOL

		Test Organisms — Gram-positive							
	Per Cent	*S-a*	*St. fec.*	*S-1*	*C. ovi.*	*E-2*	*C. hof.*	*S-3*	*S-2*
20°	36	0 0 0	...	...	...	...	...	...	...
	34	0/+0 0	0 0 0	0 0 0	0 0 0	...	...	...	...
	32	+ 0/+0	+0/+0	+0 0	+0 0	0 0 0	0 0 0	0 0 0	...
	30	+ + +	++ +	+++	++0	+ +0	++0	+0/+0	0 0 0
	28	...	...	...	+++	+ ++	+++	++ 0/+	+0/+0
	26	...	...	...	...	...	...	++ +	++ +
No. of tests		5	4	4	4	5	3	5	5
37°	24	...	0 0 0	0 0 0	...	0 0 0	...	...	...
	22	0 0 0	+0 0	+0 0	0 0 0	0/+0 0	0 0 0	...	0 0 0
	20	+ + 0	++ 0	+++	+0 0	+ 0 0	+0 0	...	+0 0
	18	+ + +	++ +	...	+++	+ ++	+++	0 0 0	++ +
	16	...	...	...	...	...	...	+0 0	...
	14	...	...	...	...	...	...	++ +	...
No. of tests		5	5	4	4	5	5	5	5

(*Cont'd on p. 101*)

TABLE VII.—ISOPROPYL ALCOHOL—(*Cont'd from p. 100*)

		Test Organisms — Gram-negative					
	Per Cent	*S. mar.*	*E. col.*	*Ps. aer.*	*A. aer.*	*P. vul.*	*E. typhosa*
20°	30	0 0 0	...	...	...	...	...
	28	++0	0 0 0	0 0 0	0 0 0	0 0 0	...
	26	+++	++ +	+0/+0	+0 0	0/+0 0	0 0 0
	24	...	...	++ +	+++	+ + +	+++
No. of tests		5	5	5	4	5	5
37°	18	0 0 0	0 0 0	...	...	...	...
	16	+0 0	+0/+0	0 0 0	0 0 0	0 0 0	0 0 0
	14	+++	++ +	++ 0	+0 0	+ 0/+0	+0 0
	12	...	...	++ +	+++	+ + +	+++
No. of tests		5	5	5	4	5	5

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE VIII.—CRESOL (U. S. P.)

		Test Organisms — Gram-positive							
	Diln.	*S-2*	*S-1*	*E-2*	*S-a*	*St. fec.*	*C. ovi.*	*C. hof.*	*S-3*
20°	1:50	0 0 0	...	...	...	...	...	...	...
	1:60	++0/+	...	...	...	...	...	...	...
	1:70	+++	...	...	...	...	...	...	...
	1:80	...	0 0 0	0 0 0	...	...	...	...	...
	1:90	...	+0 0	+0 0	...	...	...	...	...
	1:100	...	++ 0	+0/+0	0 0 0	0 0 0	0 0 0	0 0 0	...
	1:150	...	++ +	++ +	+0/+0	+ 0 0	0/+0 0	0/+0 0	...
	1:200	...	...	...	++ +	+ + +	+ + +	+ + +	...
	1:500	...	...	...	...	...	...	...	0/+0 0
	1:600	...	...	...	...	...	...	...	+ 0/+0
	1:700	...	...	...	...	...	...	...	+ + 0
	1:800	...	...	...	...	...	...	...	+ + +
No. of tests		6	7	6	6	5	5	6	7
37°	1:90	+0 0	...	...	...	...	...	...	...
	1:100	++0	...	...	...	...	...	...	...
	1:150	+++	...	0 0 0	...	0 0 0	...	...	...
	1:200	...	+0/+0	+0 0	...	0/+0 0	...	...	...
	1:250	...	++ 0/+	++ 0/+	0 0 0	+ 0/+0	0 0 0	0 0 0	...
	1:300	...	++ +	++ +	+0/+0	+ + +	+ 0/+0	+ 0/+0	...
	1:350	...	...	...	++ 0	...	+ + +	+ + 0/+	...
	1:400	...	...	...	++ +	...	...	+ + +	...
	1:800	...	...	...	...	...	...	...	0 0 0
	1:1000	...	...	...	...	...	...	...	0/+0 0
	1:1200	...	...	...	...	...	...	...	+ 0/+0
	1:1400	...	...	...	...	...	...	...	+ + 0
	1:1600	...	...	...	...	...	...	...	+ + +
No. of tests		7	7	7	6	6	5	6	8

		Gram-negative					
	Diln.	*E. col.*	*A. aer.*	*E. typhosa*	*S. mar.*	*Ps. aer.*	*P. vul.*
20°	1:80	0 0 0	0 0 0	...	...	...	...
	1:90	+0 0	0/+0 0	...	...	...	...
	1:100	+0 0	+ 0 0	0 0 0	0 0 0	0 0 0	...
	1:150	++ +	+ + 0	+0 0	+0 0	+0 0	0 0 0
	1:200	...	+ + +	+++	+++	+++	++ +
No. of tests		4	4	4	4	6	5
37°	1:150	0 0 0	...	...	...	0 0 0	0 0 0
	1:200	+0/+0	0 0 0	...	0 0 0	+0 0	0/+0 0
	1:250	++ +	+ 0/+0	0 0 0	+0 0	+++	+ +0/+
	1:300	...	+ + +	++0/+	+++	...	+ ++
	1:350	...	...	+++	...	...	...
No. of tests		5	5	5	5	6	7

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE IX.—HYDROCHLORIC ACID

	Diln. of 36.3% Soln.	Conc. Gm./Ml. HCl	Gram-positive: *St. fec.*	*S-a*	*S-1*	*S-2*	*C. ovi.*	*S-3*	*E-2*	*C. hof.*
20°	1:40	1:93	0 0 0	...	...	..	...	...	...	...
	1:50	1:116	+0 0	0 0 0	...	...	...	...	...	...
	1:60	1:140	+0 0	+0 0	...	...	...	...	...	...
	1:70	1:163	+0/+0	+0 0	0 0 0	...	...	...	...	...
	1:80	1:186	++ 0	+0/+0/+	0/+0	...	...	...	...	...
	1:90	1:210	++ 0	++ +	0/+0 0	...	...	...	...	...
	1:100	1:233	++ 0	. .	0/+0 0	0 0 0 0 0 0		...	...	...
	1:150	1:350	++ +	...	+ 0 0	+0 0 0/+0 0	0 0 0	0 0 0		...
	1:200	1:467	...	..	+ ++	+0/+0 + 0/+0/+	0/+0 0	0/+0 0	0 0 0	
	1:250	1:584	...		...	++ + + + 0/+	+ 0/+0	+ 0 0	+ 0/+0/+	
	1:300	1:700	...	..	...	... + + +	+ 0/+0	+ 0/+0	+ ++	
	1:350	1:817	...	...	...	...	... + + 0/+	+ 0/+0	...	
	1:400	1:934	...	...	...	...	... + + +	+ + 0/+	...	
	1:500	1:1168	...	...	...	...	...	... + + +	...	
No. of tests			5	8	8	7	8	10	12	10
37°	1:100	1:233	. .	0 0 0	0 0 0	. .	...	...	...	...
	1:150	1:350	...	+0 0	...	...	..	...	...	...
	1:200	1:467	...	++0/+	0/+0 0	...	...	...	...	...
	1:250	1:584	0 0 0	+++	...	...	...	...	...	...
	1:300	1:700	0/+0 0	...	+ 0 0	...	...	...	...	...
	1:350	1:817	+ 0 0	..	...	...	...	...	...	...
	1:400	1:934	+ 0/+0/+	..	+ 0 0	0 0 0 0 0 0	...	0 0 0	0 0 0	0 0 0
	1:500	1:1168	+ + +	..	+ 0/+0	+0 0 0/+0 0	...	0/+0 0	0/+0 0	
	1:600	1:1401	...	...	+ + 0	+0/+0 0/+0 0	...	0/+0 0	0/+0/+0	
	1:700	1:1635	. .	...	+ + +	++ + + 0 0	0 0 0	+ 0 0	+ 0/+0	
	1:800	1:1869	..	..	...	... + + 0/+	+ 0 0	+ 0 0	+ + +	
	1:900	1:2102	...	...	. .	.. + + +	+ 0/+0	+ + 0	...	
	1:1000	1:2336	...	...	...	...	... + 0/+0	+ + 0	...	
	1:1100	1:2570	...	...	. .	. .	...	...	+ + 0/+	...
	1:1200	1:2803	..	...	...	...	...	+ + 0	+ + +	...
	1:1300	1:3037	...	...	...	...	...	+ + +	...	...
No. of tests			8	6	7	7	11	11	9	8

	Diln. of 36.3% Soln.	Conc. Gm./Ml. HCl	Gram-negative: *P. vul.*	*E col.*	*A. aer*	*S. mar.*	*E. typhosa*	*Ps. aer.*
20°	1:80	1:186	0 0 0	..	...	...	...	...
	1:90	1:210	0/+0 0	0 0 0	..	...	...	...
	1:100	1:233	0/+0 0	0/+0 0	...	...	...	...
	1:150	1:350	0/+0/+0	+ 0/+0	0 0 0	0 0 0	0 0 0	...
	1:200	1:467	+ 0/+0	+ + +	0/+0/+0	0/+0 0	0/+0 0	....
	1:250	1:584	+ + 0/+	...	+ 0/+0	...	...	...
	1:300	1:700	+ + +	...	+ + 0	+ 0/+0	+ 0 0	...
	1:350	1:817	...	...	+ + +	...	...	...
	1:400	1:934	...	...	...	+ + 0/+	+ 0/+0/+	0 0 0
	1:500	1:1168	...	...	...	+ + +	+ + +	0/+0 0
	1:600	1:1401	...	...	...	...	...	0/+0 0
	1:700	1:1635	...	...	...	...	...	0/+0/+0
	1:800	1:1869	...	...	...	...	...	+ 0/+0/+
	1:900	1:2102	...	...	...	...	...	+ + 0/+
	1:1000	1:2336	...	...	...	...	...	+ + +
No. of tests			11	7	11	8	14	13
37°	1:90	1:210	...	0 0 0	...	...	...	...
	1:100	1:233	...	0/+0 0	...	...	...	...
	1:150	1:350	...	0/+0 0	...	...	...	...
	1:200	1:467	...	+ 0/+0/+	...	...	...	...
	1:250	1:584	...	+ + +	...	...	...	...
	1:300	1:700	0 0 0	..	0 0 0	...	...	...
	1:400	1:934	0/+0 0	...	0/+0/+0	0 0 0	...	...
	1:500	1:1168	+ 0/+0	...	0/+0/+0	0/+0 0	...	...
	1:600	1:1401	+ 0/+0	...	0/+0/+0	0/+0/+0	0 0 0	...
	1:700	1:1635	+ + 0	...	+ 0/+0/+	+ 0/+0	+ 0 0	...
	1:800	1:1869	+ + +	...	+ + +	+ 0/+0/+	+ 0/+0	...
	1:900	1:2102	...	...	...	+ + +	+ + 0/+	...
	1:1000	1:2336	...	...	...	...	+ + +	0 0 0
	1:1100	1:2570	...	...	...	...	...	0/+0/+0
	1:1200	1:2803	...	...	...	...	...	0/+0/+0
	1:1300	1:3037	...	...	...	...	...	+ + +
No. of tests			11	7	11	12	14	10

TABLE X.—SODIUM HYDROXIDE

(18.9 *N*—Sat. Sol.)

	Diln. of Sat. Solu.	Conc. Gm./Ml. NaOH	Gram-positive: *S-2*	*C. hof.*	*S-a*	*C. ovi.*	*E-2*	*St. fec.*	*S-1*	*S-3*
20°	1:10	1:13.2	0 0 0	. .	. .	. . .	. . .	. . .	. . .	. . .
	1:20	1:26.5	0/+0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	. .
	1:30	1:39.7	+ 0/+0	+0/+ 0/+	+0/+0/+	0/+0 0	0/+0 0	0/+0 0	0/+0 0	. . .
	1:40	1:52.9	+ + +	++ +	++ +	+ ++	+ + 0/++	0/+0	+ 0 0	. . .
	1:50	1:66.2	. .	. .	.		.	+ 0/+0	+ +0/+	. .
	1:60	1:79.4	. . .	.	.		+ + 0/++	0/+0/+	+ ++	.
	1:70	1:92.6	. .	.		.	+ + + +	+ +	.	. .
	1:90	1:119.1	. . .	. . .	.	. .	. .	. . .	. .	0 0 0
	1:100	1:132.2	.	. . .		. . .	. .	. .	. .	0/+0 0
	1:200	1:264.4	. .	. . .	.	. .	. .	. .	.	+ ++
No. of tests			6	6	9	5	6	6	6	6
37°	1:30	1:39.7	.	0 0 0	.	. .	. . .	. .	.	. .
	1:40	1:52.9	0 0 0	+0 0		.	0 0 0	0 0 0	. .	.
	1:50	1:66.2	0/+0 0	+0 0	. .	0 0 0	0/+0 0	0/+0 0	0 0 0	.
	1:60	1:79.4	+ 0/+0	+0/+0/+	0 0 0	+0/+ 0/+	0/+0 0	0/+0 0	0/+0 0	.
	1:70	1:92.6	+ 0/+0/+	++ 0/+	++ 0	+0/+ 0/+	+ 0 0	+ 0/+0/+	0/+0 0	.
	1:80	1:105.8	+ 0/+0/+		++ 0	+0/+ 0/+	+ + +	+ + +	+ 0 0	
	1:90	1:119.1	+ + +	++ 0/+	++ +	++ 0/+	.	. . .	+ +0	. .
	1:100	1:132.2	. .	++ +	.	++ +	. .	. . .	+ +0/+	0 0 0
	1:200	1:264.4	. .	.	.	. .	. . .	. . .	+ ++	+ +0
	1:300	1:396.6	. . .	. .		. . .	. .	. . .	. .	+ ++
No. of tests			6	6	8	5	7	5	5	6

	Diln. of Sat. Soln.	Conc of NaOH	Gram-negative: *P. vul.*	*A. aer.*	*S. mar.*	*Ps. aer.*	*E. typhosa*	*E. col.*
20°	1:200	1:264.4	+ 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	1:300	1:396.6	+ +0	+ +0/+	+0/+0/+	+0/+0	+0/+ 0	0/+0/+0
	1:400	1:528.8	+ +0/+	+ ++	++ 0/+	++ +	++ +	+ + +
	1:500	1:662.0	+ ++	. . .	++ +	. .		
No. of tests			7	6	6	6	7	7
37°	1:100	1:132.2	. . .	0 0 0	. . .	.	.	
	1:200	1:264.4	0 0 0	0/+0 0	.	0 0 0	.	0 0 0
	1:300	1:396.6	0/+0/+0	+ 0 0	0 0 0	0/+0 0	0 0 0	0/+0 0
	1:400	1:528.8	+ + 0	+ +0/+	+0 0	+ +0	0/+0 0	+ + 0/+
	1:500	1:662.0	+ + +	+ ++	++ 0/+	+ ++	+ + 0/+	+ + +
	1:600	1:793.2	. . .	. . .	++ +		+ + 0/+	.
	1:700	1:925.4	. .	. .	. . .	.	+ + +	
No. of tests			6	7	6	6	8	7

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE XI.—IODINE

	Diln.	Gram-positive: *C. hof.*	*S-1*	*St. fec.*	*C. ovi.*	*S-a*	*E-2*	*S-3*	*S-2*
20°	1:5000	0 0 0	0 0 0	. . .	. . .	. .			
	1:6000	0/+0 0	0/+0 0	0 0 0	.	.	. . .	. .	
	1:7000	+ +0/+	0/+0 0	+0 0	0/+0 0	0 0 0	. .	. . .	
	1:8000	+ + +	+ + +	+++	+ + +	++ +	0 0 0	. .	
	1:9000	. . .	. . .	. . .	. . .		0/+0/+0/+	.	
	1:10,000	. . .	. . .	. . .	.	.	+ + +	0 0 0	0 0 0
	1:11,000	.	. . .	. . .	. .	. . .	. . .	0/+0/+0/+	0/+0/+0
	1:12,000	. .	. . .	. . .			. . .	+ + +	0/+0/+0
	1:13,000	. .	. . .	. . .	. . .	. .	. . .	. . .	+ + 0/+
	1:14,000	. . .	. . .	. . .	. . .	. .	. . .	. . .	+ + 0/+
	1:15,000	. . .	. . .	. . .	. . .	. . .	. . .	. . .	+ + +
No. of tests		7	6	6	5	7	5	6	6

(*Cont' on p. 104*)

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE IX.—HYDROCHLORIC ACID

	Diln. of 36.3% Soln.	Conc. Gm./Ml. HCl	*St. fec.*	*S-a*	*S-1*	*S-2*	*C. ovi.*	*S-3*	*E-2*	*C. hof.*
			Test Organisms — Gram-positive							
20°	1:40	1:93	0 0 0	...	...	..	...	...	...	...
	1:50	1:116	+0 0	0 0 0	...	...	...	...	...	...
	1:60	1:140	+0 0	+0 0	...	...	...	...	...	...
	1:70	1:163	+0/+0	+0 0	0 0 0	...	...	...	...	...
	1:80	1:186	++ 0	+0/+0/+	0/+0	...	...	...	...	...
	1:90	1:210	++ 0	++ +	0/+0 0	...	...	...	...	...
	1:100	1:233	++ 0	...	0/+0 0	0 0 0 0 0 0	...	...	...	...
	1:150	1:350	++ +	..	+ 0 0	+0 0 0/+0 0	0 0 0	0 0 0		...
	1:200	1:467	...	.	++ ++	+0/+0 + 0/+0/+	0/+0 0	0/+0 0	0 0 0	
	1:250	1:584	...	..	...	++ + + + 0/+	+ 0/+0	+ 0 0	+ 0/+0/+	
	1:300	1:700	...	...	...	... + + +	+ 0/+0	+ 0/+0	+ ++	
	1:350	1:817	...	...	..	...	.. + + 0/+	+ 0/+0	...	
	1:400	1:934	...	...	...	...	... + + +	+ + 0/+	...	
	1:500	1:1168	...	...	...	...	...	... + + +	...	
No. of tests			5	8	8	7	8	10	12	10
37°	1:100	1:233	...	0 0 0	0 0 0	...	...	...	...	...
	1:150	1:350	...	+0 0	..	...	..	...	...	...
	1:200	1:467	...	++0/+	0/+0 0	...	...	...	...	...
	1:250	1:584	0 0 0	+++	...	...	...	...	...	...
	1:300	1:700	0/+0 0	..	+ 0 0	...	...	...	...	...
	1:350	1:817	+ 0 0	...	...	...	...	...	...	...
	1:400	1:934	+ 0/+0/+	..	+ 0 0	0 0 0 0 0 0	...	0 0 0	0 0 0	
	1:500	1:1168	+ + +	...	+ 0/+0	+0 0 0/+0 0	...	0/+0 0	0/+0 0	
	1:600	1:1401	...	...	+ + 0	+0/+0 0/+0 0	...	0/+0 0	0/+0/+0	
	1:700	1:1635	..	..	+ + +	++ + + 0 0	0 0 0	+ 0 0	+ 0/+0	
	1:800	1:1869	..	..	...	... + +	0/+ + 0 0	+ 0 0	+ + +	
	1:900	1:2102	...	...	...	... + + +	+ 0/+0	+ + 0	...	
	1:1000	1:2336	...	...	...	.. ...	+ 0/+0	+ + 0	...	
	1:1100	1:2570	..	...	...	... ...	...	+ + 0/+	...	
	1:1200	1:2803	...	...	...	... ...	+ + 0	+ + +	...	
	1:1300	1:3037	...	...	...	... ...	+ + +	...	...	
No. of tests			8	6	7	7	11	11	9	8

	Diln. of 36.3% Soln.	Conc Gm./Ml. HCl	*P. vul.*	*E col.*	*A. aer*	*S. mar.*	*E. typhosa*	*Ps. aer.*
			Gram-negative					
20°	1:80	1:186	0 0 0	..	...	...	...	...
	1:90	1:210	0/+0 0	0 0 0	.	...	...	...
	1:100	1:233	0/+0 0	0/+0 0	...	...	...	...
	1:150	1:350	0/+0/+0	+ 0/+0	0 0 0	0 0 0	0 0 0	...
	1:200	1:467	+ 0/+0	+ + +	0/+0/+0	0/+0 0	0/+0 0	...
	1:250	1:584	+ + 0/+	...	+ 0/+0	...	...	...
	1:300	1:700	+ + +	...	+ + 0	+ 0/+0	+ 0 0	...
	1:350	1:817	...	...	+ + +	...	...	...
	1:400	1:934	...	...	...	+ + 0/+	+ 0/+0/+	0 0 0
	1:500	1:1168	...	...	...	+ + +	+ + +	0/+0 0
	1:600	1:1401	...	...	...	...	...	0/+0 0
	1:700	1:1635	...	...	..	...	...	0/+0/+0
	1:800	1:1869	...	...	...	...	...	+ 0/+0/+
	1:900	1:2102	...	...	...	...	...	+ + 0/+
	1:1000	1:2336	...	...	...	...	...	+ + +
No. of tests			11	7	11	8	14	13
37°	1:90	1:210	...	0 0 0	...	...	...	...
	1:100	1:233	...	0/+0 0	...	...	...	...
	1:150	1:350	...	0/+0 0	...	...	...	...
	1:200	1:467	...	+ 0/+0/+	...	...	...	...
	1:250	1:584	...	+ + +	...	...	...	...
	1:300	1:700	0 0 0	...	0 0 0	...	...	...
	1:400	1:934	0/+0 0	...	0/+0/+0	0 0 0	...	...
	1:500	1:1168	+ 0/+0	...	0/+0/+0	0/+0 0	...	...
	1:600	1:1401	+ 0/+0	...	0/+0/+0	0/+0/+0	0 0 0	...
	1:700	1:1635	+ + 0	...	+ 0/+0/+	+ 0/+0	+ 0 0	...
	1:800	1:1869	+ + +	...	+ + +	+ 0/+0/+	+ 0/+0	...
	1:900	1:2102	...	...	...	+ + +	+ + 0/+	...
	1:1000	1:2336	...	...	...	...	+ + +	0 0 0
	1:1100	1:2570	...	...	...	...	...	0/+0/+0
	1:1200	1:2803	...	...	...	...	...	0/+0/+0
	1:1300	1:3037	...	...	...	...	...	+ + +
No. of tests			11	7	11	12	14	10

TABLE X.—SODIUM HYDROXIDE

(18.9 *N*—Sat. Sol.)

	Diln. of Sat. Soln.	Conc. Gm./Ml. NaOH	*S-2*	*C. hof.*	*S-a*	*C. ovi.*	*E-2*	*St. fec.*	*S-1*	*S-3*
			TEST ORGANISMS—Gram-positive							
20°	1:10	1:13.2	0 0 0	...	...	...	...	...	...	...
	1:20	1:26.5	0/+0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	...
	1:30	1:39.7	+ 0/+0	+0/+ 0/+	+0/+0/+	0/+0 0	0/+0 0	0/+0 0	0/+0 0	...
	1:40	1:52.9	+ + +	++ +	++ +	+ ++	+ + 0/++	0/+0	+ 0 0	...
	1:50	1:66.2	.	...	...	. .	.	+ 0/+0	+ +0/+	...
	1:60	1:79.4	...	.	.	...	+ + 0/++	0/+0/+	+ ++	...
	1:70	1:92.6	.	.		...	+ + + +	+ +	...	...
	1:90	1:119 1	.	...	. .	...	...	...	...	0 0 0
	1:100	1:132.2		...	. .	...	...	...	...	0/+0 0
	1:200	1:264.4	.	...	. .	. .	...	...	...	+ ++
No. of tests			6	6	9	5	6	6	6	6
37°	1:30	1:39.7		0 0 0	..	...	...	...	...	...
	1:40	1:52.9	0 0 0	+0 0	.	..	0 0 0	0 0 0	...	...
	1:50	1:66.2	0/+0 0	+0 0	. .	0 0 0	0/+0 0	0/+0 0	0 0 0	...
	1:60	1:79.4	+ 0/+0	+0/+0/+	0 0 0	+0/+ 0/+	0/+0 0	0/+0 0	0/+0 0	...
	1:70	1:92.6	+ 0/+0/+	++ 0/+	++ 0	+0/+ 0/+	+ 0 0	+ 0/+0/+	0/+0 0	...
	1:80	1:105.8	+ 0/+0/+	.	++ 0	+0/+ 0/+	+ + +	+ + +	+ 0 0	...
	1:90	1:119 1	+ + +	++ 0/+	++ +	++ 0/+	...	...	+ +0	...
	1:100	1:132 2		++ +	.	++ +	...	...	+ +0/+	0 0 0
	1:200	1:264.4	. .	.	.	.	...	...	+ ++	+ +0
	1:300	1:396.6	..	...		..	..	...	...	+ ++
No. of tests			6	6	8	5	7	5	5	6

	Diln. of Sat. Soln.	Conc of NaOH	*P. vul.*	*A. aer*	*S. mar.*	*Ps. aer.*	*E. typhosa*	*E. col.*
			Gram-negative					
20°	1:200	1:264.4	+ 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	1:300	1:396.6	+ +0	+ +0/+	+0/+0/+	+0/+0	+0/+ 0	0/+0/+0
	1:400	1:528 8	+ +0/+	+ ++	++ 0/+	++ +	++ +	+ + +
	1:500	1:662.0	+ ++	..	++ +	...	...	...
No. of tests			7	6	6	6	7	7
37°	1:100	1:132.2	...	0 0 0	...	...	...	...
	1:200	1:264.4	0 0 0	0/+0 0	.	0 0 0	...	0 0 0
	1:300	1:396.6	0/+0/+0	+ 0 0	0 0 0	0/+0 0	0 0 0	0/+0 0
	1:400	1:528.8	+ + 0	+ +0/+	+0 0	+ +0	0/+0 0	+ + 0/+
	1:500	1:662.0	+ + +	+ ++	++ 0/+	+ ++	+ + 0/+	+ + +
	1:600	1:793.2	..	..	++ +	. .	+ + 0/+	...
	1:700	1:925.4	.	..	...	...	+ + +	...
No. of tests			6	7	6	6	8	7

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE XI.—IODINE

	Diln.	*C. hof.*	*S-1*	*St. fec.*	*C. ovi.*	*S-a*	*E-2*	*S-3*	*S-2*
		TEST ORGANISMS—Gram-positive							
20°	1:5000	0 0 0	0 0 0	.	.	..	...	...	...
	1:6000	0/+0 0	0/+0 0	0 0 0	.	...	...	...	...
	1:7000	+ +0/+	0/+0 0	+0 0	0/+0 0	0 0 0	...	...	...
	1:8000	+ + +	+ + +	+++	+ + + ++	+ 0 0 0	...	...	...
	1:9000	...	...	...	...	..	0/+0/+0/+	...	..
	1:10,000	.	...	...		. .	+ + +	0 0 0	0 0 0
	1:11,000		..	..	.	...	...	0/+0/+0/+	0/+0/+0
	1:12,000	.	. .	. .	.	..	...	+ + +	0/+0/+0
	1:13,000	.	. .	..	...	..	...	...	+ + 0/+
	1:14,000	...	...	...	...	.	...	...	+ + 0/+
	1:15,000	..	.	...		..	...	...	+ + +
No. of tests		7	6	6	5	7	5	6	6

(*Cont' on p. 104*)

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE XI.—IODINE—(*Cont'd from p. 103*)

		Test Organisms							
		Gram-positive							
	Diln.	*C. hof.*	*S-1*	*St. fec.*	*C. ovi.*	*S-a*	*E-2*	*S-3*	*S-2*
37°	1:5000	0 0 0	...	...	...	...	...	...	...
	1:6000	0/+0 0 0 0 0		...	...	...	...	...	...
	1:7000	+ +0/+ +0/+	0/+ 0 0 00		0 0 0 0 0		...	...	...
	1:8000	+ + + ++	+	+++	0/+0/+0/+	0/+0/+0/+	0 0 0	...	...
	1:9000	...	...	...	+ + +	+ + +	+0/+ 0	...	...
	1:10,000	...	...	...	...	...	++ +	...	0 0 0
	1:11,000	...	...	...	...	...	...	0 0 0	0/+0/+0
	1:12,000	...	...	...	...	...	...	0/+0/+0/+	0/+0/+0/+
	1:13,000	...	...	...	...	...	...	+ + +	+ + 0/+
	1:14,000	...	...	...	...	...	...	...	+ + 0/+
	1:15,000	...	...	...	...	...	...	...	+ + +
No. of tests		6	6	5	5	7	6	6	7

		Gram-negative					
	Diln.	*Ps. aer.*	*A. aer.*	*E. col.*	*S. mar.*	*E. typhosa*	*P. vul.*
20°	1:5000	0 0 0	...	...	...	...	...
	1:6000	+ + +	0 0 0	...	...	...	...
	1:7000	...	0/+0/+0/+	0 0 0	0 0 0	0 0 0	...
	1:8000	...	+ + +	+ + +	++ +	0/+0 0	...
	1:9000	...	...	...	...	+ + +	0 0 0
	1:10,000	...	...	...	...	...	0/+0 0
	1:11,000	...	...	...	...	...	+ + +
No. of tests		5	5	5	6	7	6
37°	1:4000	0 0 0	...	...	...	...	...
	1:5000	+ + +	...	0 0 0	...	...	...
	1:6000	..	0 0 0	+ + +	0 0 0	0 0 0	...
	1:7000	...	0/+0/+0/+	...	0/+0/+0/+	0/+0/+0/+	...
	1:8000	...	0/+0/+0/+	...	+ + +	+ + +	0 0 0
	1:9000	...	+ + +	...	...	...	0/+0 0
	1:10,000	...	...	..	...	...	0/+0 0
	1:11,000	...	...	...	...	...	+ 0/+0
	1:12,000	. .	...	...	...	...	+ + +
No. of tests		6	6	5	5	6	6

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE XII.—HYDROGEN PEROXIDE

		Test Organisms							
		Gram-positive							
	Per Cent	*S-1*	*St. fec.*	*C. ovi.*	*C. hof.*	*E-2*	*S-a*	*S-3*	*S-2*
20°	12	+0 0	...	...	...	...	...	...	...
	11	+0 0	...	...	...	...	...	...	...
	10	+0 0	0 0 0	0 0 0	0 0 0	...	...	...	...
	9	++0	0/+0 0	0/+0 0	0/+0 0	...	...	...	...
	8	+++	+ 0 0	+ 0 0	0/+0 0	...	...	...	...
	7	...	+ 0 0	+ 0 0	+ 0 0	0 0 0 0 0 0		...	...
	6	...	+ + 0	+ 0/+0	+ 0/+0	+0 0 0/+0 0		0 0 0	0 0 0
	5	...	+ ++	+ + +	+ + 0/+	+0 0 + 0 0		+0 0	0/+0 0
	4	...	...	...	+ + +	+0 0 + +0/+		+0 0	+ 0 0
	3	...	...	...	...	++0 + ++		++0/+	+ 0/+0
	2	...	...	...	...	+++	...	+++	+ + +
No. of tests		7	9	7	7	7	6	7	5
37°	7	0 0 0	...	...	...	...	...	...	...
	6	+0 0	0 0 0	...	...	...	...	...	...
	5	+0/+0	0/+0 0	0 0 0	0 0 0	...	...	...	...
	4	++ 0	+ 0 0	0/+0 0	+0 0	...	...	...	...
	3.5	...	...	...	...	...	0 0 0	...	...
	3	++ 0/+	+ 0/+0	0/+0 0	+0 0	0 0 0	0/+0 0	0 0 0	...
	2.5	...	...	...	...	...	+ 0 0	0/+0 0	0 0 0
	2	++ +	+ + 0/+	+ 0/+0	++ 0	+0 0	+ +0	+ 0 0	+0 0
	1.5	...	...	...	...	++0	+ ++	+ 0 0	+0/+ 0
	1	...	+ + +	+ + 0/+	++ +	+++	...	+ 0 0	++ 0/+
	0.5	...	...	+ + +	...	...	...	+ ++	++ +
No. of tests		6	8	7	7	6	6	7	5

(*Cont'd on p. 105*)

TABLE XII.—HYDROGEN PEROXIDE—(*Cont'd from p. 104*)

		TEST ORGANISMS — Gram-negative					
	Per Cent	*S. mar.*	*A. aer.*	*Ps. aer.*	*P. vul.*	*E. col.*	*E. typhosa*
20°	10	0 0 0	...	...	...	...	...
	9	+0/+0	...	...	...	...	...
	8	++ +	...	...	...	...	...
	4	...	0 0 0	...	...	...	...
	3	...	+0 0	0 0 0	0 0 0	...	...
	2.5	...	...	+0 0	0/+0 0	0 0 0	...
	2	...	+0 0	+0/+ 0	+ 0 0	+0 0	0 0 0
	1.5	...	...	++ +	+ +0/+	+0 0	0/+0 0
	1	...	+++	...	+ ++	+0/+0	+ +0/+
	0.5	...	...	...	...	++ +	+ ++
No. of tests		8	8	6	7	7	7
37°	7	0 0 0	...	...	...	...	...
	6	+0 0	...	...	...	...	...
	5	+0 0	...	...	...	...	...
	4	+0/+0	...	...	...	...	
	3	++ 0	...	...	...	...	...
	2	++ 0/+	...	0 0 0	...	...	...
	1.5	++ +	0 0 0	0/+0 0	0 0 0	...	...
	1	...	0/+0 0	+ 0 0	0/+0 0	0 0 0	0 0 0
	0.5	...	+ 0/+0	+ ++	+ +0/+	0/+0 0	0/+0 0
	0.4	...	+ + 0/+	...	+ ++	+ 0 0	+ 0 0
	0.3	...	+ + +	...	...	+ 0/+0	+ 0/+ 0
	0.2	...	...	...	...	+ + 0/+	+ + 0
	0.1	...	...	...	...	+ + +	+ + +
No. of tests		7	6	7	6	7	7

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE XIII.—SILVER NITRATE

		TEST ORGANISMS — Gram-positive						
	Diln.	*C. ovi.*	*St. fec.*	*C. hof.*	*S-1*	*E-2*	*S-a*	*S-2*	*S-3*
20°	1:500	0 0 0	0 0 0	0 0 0	...	...	...	...	...
	1:1000	0/+0 0	0/+0 0	0/+0 0	0 0 0	0 0 0	...	...	...
	1:2000	0/+0 0	0/+0 0	0/+0 0	+0/+ 0	0/+0 0	0 0 0	...	...
	1:4000	+ 0 0	+ 0 0	+ 0 0	++ 0/+	0/+0 0	0/+0 0 0	0 0	...
	1:6000	+ 0/+0/+	+ +0	+ 0/+0	++ +	+ 0 0	+ 0 0 0/+0	0	...
	1:8000	+ + +	+ ++	+ + 0/+	...	+ 0 0	+ 0 0 +	0 0	...
	1:10,000	...	...	+ + +	...	+ +0/+	+ + 0 +	0/+0/+ 0	0 0
	1:12,000	...	...	...	...	+ ++	+ ++ +	+ +	0/+0/+0
	1:14,000	...	...	...	...	...	...	...	+ 0/+0/+
	1:16,000	...	...	...	...	...	...	...	+ + 0/+
	1:18,000	...	...	...	...	...	...	...	+ + +
No. of tests		5	5	5	6	6	5	6	6

		Gram-negative					
	Diln.	*E. col.*	*Ps. aer.*	*P. vul.*	*A. aer.*	*S. mar.*	*E. typhosa*
20°	1:500	0 0 0	...	...	...	...	...
	1:1000	0/+0 0	0 0 0	...	...	...	...
	1:2000	0/+0 0	0/+0 0	0 0 0	0 0 0	0 0 0	0 0 0
	1:4000	+ 0/+0	+ 0 0	0/+0/+0	+0 0	+0 0	0/+0 0
	1:6000	+ + +	+ +0/+	+ 0/+0	+0/+0	+0/+0	+ 0 0
	1:8000	...	+ ++	+ + +	++ +	++ 0/+	+ 0 0
	1:10,000	...	...	...	...	++ +	+ 0/+0
	1:12,000	...	...	...	...	...	+ 0/+0
	1:14,000	...	...	...	...	...	+ + +
No. of tests		6	5	5	5	5	5

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

agent at 37°. Silver nitrate results at 37° appear in Table XIII, since results on only four organisms were obtained in this instance.

Although susceptible to the chemical reagents at higher dilutions when tested at 37°, as compared with the 20° test, a general conformity of relative resistance is demonstrated. However, in tests against every compound one or more organisms, to a greater or less degree, distorted the relationship of resistance at the two different temperatures. In contrast to the other compounds used as test reagents, critical concentrations of iodine were either equally effective at both test temperatures for the Gram-positive organisms or required stronger concentrations at the higher temperature for the Gram-negative bacteria. Also, it should be noted that germicidal efficiency of a compound cannot be compared with another on a dilution basis. For example, although the mercury compounds were utilized at weak dilutions, the generally nongermicidal results were nonetheless based upon the maximum concentrations obtainable in solution. The two alcohols, on the other hand, while requiring relatively strong concentrations to effect germicidal action, not only killed all the test organisms within a narrow range of dilution, but could be utilized at much greater concentrations should necessity demand. The general utility as germicides of the two latter compounds, therefore, far exceeds that of the three mercury products subjected to test. The germicidal impotency of the mercurial substances prevented their use even for detecting relative resistance among the organisms. The differences in reaction of the organisms to the alcohols fell within such a narrow range of effective dilutions that these substances are not particularly suitable for determining relative resistance. On the contrary, there was marked difference in the reactions of the various organisms to hydrochloric acid. No two organisms succumbed to the same concentration; and the frequency with which the individual strains survived a wide range of dilutions at the different time intervals renders this compound a selective agent worthy of consideration. In contrast, was the reaction of the organisms to sodium hydroxide. Within both the Gram-positive and Gram-negative groups there was, in general, very little difference in resistance. However, the Gram-negative organisms elicited the striking characteristic of succumbing to dilutions of this compound far weaker than the concentrations generally withstood by the Gram-positive group.

DISCUSSION

The germicidal values and the apparent relationship of individual resistance of strains found in this study were obtained only under the circumstances peculiar to the arbitrary conditions employed. The use of any other method of test or change of media would have resulted in different values and different ratios of resistance. Had any compound other than phenol been employed for selecting the test organisms, a different choice of strains would probably have resulted. This is apparent from the changing resistance relationship among the four closely related species of staphylococci on their exposure to different types of reagents. Likewise, exposure of the different organisms to two closely related compounds results in a rearrangement of the order of resistance. The reactions of the Gram-negative bacteria to mercuric chloride and phenyl mercuric acetate and changes in the relative reactions of the organisms to ethyl and isopropyl alcohol illustrate this.

The results emphasize again the inadequacy of the conventional phenol coefficient test to indicate the selectivity or nonselectivity of a germicide. It can be noted that the effective concentration of a compound might vary as much as tenfold, depending upon the organism used in the test. It may appear that use of a phenol coefficient type of test in this work, with certain arbitrary features, detracts from the value of the results. However, as an indication of the unpredictable resistance of various organisms, it was essential that a consistent method of treatment, both in the selection and testing, be used throughout. The procedure chosen seemed to offer the most convenient means of obtaining a considerable amount of data. Its employment does not imply its endorsement as a germicidal test, or that phenol is a desirable selective reagent; in fact, the results indicate the reverse. The most significant deduction to be gained from the accumulated data is the impossibility of adequately determining germicidal activity with one representative test organism by a single standardized type of test. If so much is evident from *in vitro* tests of germicidal potentiality, it becomes acutely manifest that the multiplicity of factors involved in antiseptic efficacy precludes the use of standard methods except for crude preliminary indications of antibacterial possibilities.

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Digitalis. VIII. The Baljet Reaction, Digitoxin and Digitoxigenin*

By FREDERICK K. BELL and JOHN C. KRANTZ, JR.

The Baljet reagent has been used in a quantitative study of the color reaction of digitoxigenin and digitoxin with that reagent. The results reported in this paper indicate the reactivity of the two substances with the Baljet reagent on a molar basis to be essentially the same. If extreme precision is required, however, correction must be made for the effect of the digitoxose portion of the digitoxin molecule.

IN BALJET'S original publication (1) of his color reaction for cardiotonic glycosides he included the observation that the genins also give a positive response to the reaction. This observation gave strong support to his conclusion that the reaction depended upon the structure of the genin portion of the glycoside, especially since he had shown that the sugars themselves gave practically no response under the conditions of the test.

In the course of our studies concerning the quantitative determination of the pure digitalis glycosides (2, 3) by means of our adaptation of the Baljet reaction, we have felt that quantitative data concerning the behavior of the genins would afford useful information concerning the validity of the method and the mechanism of the reactions involved. This seemed especially desirable since the only quantitative data available are those of Hagemeier (4) who, moreover, employed an experimental procedure quite different from that which we have adopted. In this communication we will describe and discuss the results we have obtained in our studies of digitoxigenin.

EXPERIMENTAL

Through the kindness of Dr. E. W. McChesney of the Sterling-Winthrop Research Institute we were fortunate in obtaining a sample of digitoxigenin especially prepared for research studies. This specimen of digitoxigenin is a portion of a product the properties and preparation of which have been described elsewhere (5). For purposes of comparison we have used the U. S. P. Reference Standard Digitoxin.

The experimental method employed was essentially that previously described (6) using the Fisher Electrophotometer with the 525-millimicron green filter and the 3-cc. micro-absorption cells. In the development of the color reaction we have used two procedures: (*a*) our original method in which sodium hydroxide serves as the alkali and (*b*) our recently revised method (7) in which tetraethylammonium hydroxide is the alkali.

A stock solution of digitoxigenin in absolute methanol was prepared to contain 25 mg. per 100 cc. This solution was diluted quantitatively with absolute methanol to yield concentrations of 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, and 22.5 mg. per 100 cc. Similarly, from a stock solution of digitoxin in absolute methanol containing 50 mg. per 100 cc. we prepared dilutions containing 5, 10, 15, 20, 25, 30, and 40 mg. per 100 cc.

In Fig. 1 typical results are shown graphically by plotting the maximum color development in terms of the optical density against the concentration in mg. per 100 cc. Curves *A* and *C* were obtained from digitoxigenin and digitoxin, respectively, using tetraethylammonium hydroxide as the alkali. *B* and *D* are the corresponding curves when sodium hydroxide is used as the alkali. Confirming our earlier observations, it is seen that in the case of digitoxigenin also, the use of tetraethylammonium hydroxide in place of sodium hydroxide resulted in an increase of approximately 100% in the color intensity of the test. The four curves also show that for each of the two alkalies used the intensity of the color developed by digitoxigenin is approximately twice that of digitoxin. This factor also approximates the ratio of the molecular weights of digitoxin (765) and digitoxigenin (373).

The curves in Fig. 1 have been re-plotted after converting the concentrations into terms of micromoles per 100 cc. as shown in Fig. 2. Curve *A* represents the locus of the points obtained from digitoxin and digitoxigenin using tetraethylammonium hydroxide as the alkali. In a similar manner curve *B* was obtained when sodium hydroxide was used as the alkali in the reagent.

It is seen that in each curve up to a concentration of approximately 35 micromoles per 100 cc. the values for digitoxin and digitoxigenin do fall on the same curve within the limits of error of the experimental method employed. In the higher concentration range the divergence in the values for the two substances increases gradually with increase in concentration. At a concentration of 65 micromoles per 100 cc. the divergence is definitely beyond the limit of error of the method. It is also interesting to note that the values for digitoxin for each curve are consistently higher than those for the corresponding digitoxigenin concentrations. This observation can be readily explained on the basis of reaction between the sugar portion of the glycoside molecule and the reagent. From the limited data which we have previously published (8) concerning the behavior of digitoxose in aqueous solution with

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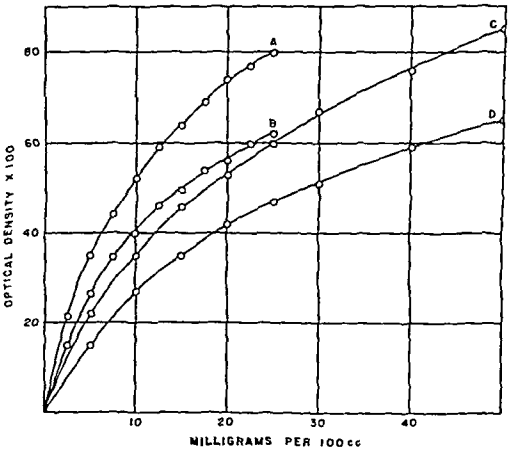

Figure 1.

A = Digitoxigenin—$(C_2H_5)_4NOH$; *B* = Digitoxigenin—NaOH; *C* = Digitoxin—$(C_2H_5)_4NOH$; *D* = Digitoxin—NaOH.

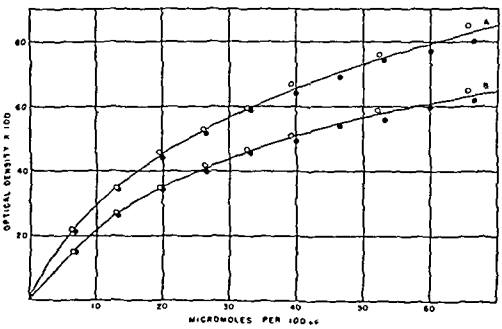

Figure 2.

A, Tetraethylammonium hydroxide as the alkali: circles = digitoxin, dots = digitoxigenin.

B, Sodium hydroxide as the alkali: circles = digitoxin, dots = digitoxigenin.

the Baljet reagent one would predict that the effect of the sugar portion of the molecule on the total color intensity would not be detectable. However, the present experiments are carried out in a strongly alcoholic medium and there is no justification in assuming that three molecules of digitoxose bound in the glycoside molecule would have the same reactivity toward the reagent as that shown in a simple aqueous solution of the sugar. Although it is clear that the sensitivity of the Baljet reaction to digitoxose is markedly less than that to the lactone structure of the glycosides, it must be remembered that the digitoxin molecule contains approximately 50% of digitoxose. Therefore in the higher concentrations of the glycoside solutions there is present the equivalent of appreciable amounts of the sugar.

In this connection it has also been suggested (9) that the reactivity of the lactone portion of the glycoside molecule is increased by the presence of the digitoxose molecules attached at C_3 of the genin. This hypothesis would appear to require that consideration be given also to the extent of possible hydrolysis of digitoxin molecule in the alkaline medium of the test solution.

CONCLUSIONS

From these observations we may draw the general conclusion that for most practical purposes the sensitivities of digitoxin and digitoxigenin on a molar basis toward the Baljet reaction are the same under the experimental conditions employed and within the concentration range examined. For more precise work and particularly in the higher concentrations a correction must be applied in the case of digitoxin to allow for the effect of the digitoxose portion of the molecule.

The significance of the behavior of digitoxigenin to the Baljet reaction with respect to our assay method for pure preparations of digitoxin is fairly obvious. Although it is doubtful that commercial preparations of pure digitoxin contain appreciable amounts of digitoxigenin, when the latter substance is present high assay values are to be expected by our method. It is generally known that the cardiotonic activity of the genin of a digitalis glycoside is usually less than that of the corresponding glycoside and this has been found to be particularly true in the case of digitoxin and digitoxigenin. Gold and his co-workers (10) have recently found that the same relationship prevails when the human assay method is employed.

If we assume that in mixtures of digitoxin and digitoxigenin the assay values obtained by both the biologic and colorimetric methods are a simple summation of the assay values for each constituent, the potency of such mixtures can be readily predicted. Thus, a sample of pure digitoxin containing 5% of digitoxigenin would probably yield a bioassay value of approximately 99%. By the colorimetric method a value of 105% would be obtained. The difference between these two values is well within the limit of reproducibility of most bioassay methods.

The data which we have presented also suggest a rather obvious and interesting speculation concerning our colorimetric assay method for digitalis preparations (11). Experience has shown that assay values obtained by that method are usually higher than those obtained by bioassay methods and frequently there are preparations which yield a much higher value by the colorimetric method. It is well known that the original glycosides in the digitalis leaf are readily broken down by enzymatic action. Does the extent or degree of such action vary with different crops of leaves and with different methods of treatment after the leaves are harvested? If we imagine the extreme case of a leaf preparation similar to the U. S. P. Reference Standard Powder in which the digitoxin is replaced by the same weight of digitoxigenin, such a product would probably yield a bioassay value of approximately 80%, while that of the colorimetric method would be 200%.

SUMMARY

The results of a quantitative study of the color reaction of digitoxigenin and digitoxin toward the Baljet reagent have been presented and discussed. It has been found that, for most practical purposes, the reactivity of the two substances on a molar basis is the same. For greater precision,

correction must be made, particularly in the higher concentrations, for the effect of the digitoxose portion of the digitoxin molecule.

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Rodent-Repellent Studies. I. Method for the Evaluation of Chemical Repellents*

By E. BELLACK and JAMES B. De WITT

A biological assay procedure and a method for the numerical expression of results have been devised for the determination of the repellency to rodents of different chemical compounds. The procedure is based upon the degree of acceptability of foods containing the candidate repellents, and has been shown to offer a rapid, reliable measure of repellent activity.

THE PRIMARY objective of the rodent-repellent studies being conducted by the Fish and Wildlife Service is the development of a treatment for paper and cardboard boxes which will prevent or minimize rodent damage to packaged goods while in storage. Such a treatment may be such that the protection is due to physical properties, such as hardness, tackiness, or grittiness, or it may be due to chemical properties, such as odor, taste, or irritancy. Examples of materials offering the first type of protection would be the relatively impervious coatings formed by waxes, plastics, or latices. Such materials might properly be termed "protectants," and will not be considered in this paper. The term "repellent," as used here, refers to those materials whose chemical characteristics are such as to cause animals to avoid contact with objects containing them.

In the initial phases of our studies it was found that little was known concerning the chemical composition of possible rodent repellents, and that it would be necessary to devise a suitable bioassay procedure permitting the rapid screening of large numbers of samples. For the purposes of simplicity of operation, the common laboratory rat was chosen as the experimental animal, and the technique described below was adopted as a standard procedure. While this technique permits the rapid screening of materials, it should be noted that it depends upon the sample coming in contact with the oral mucosa, or being ingested by the animal. Since the rat is able to gnaw through paper or cardboard without ingesting the excised particles, or even allowing them to come into contact with the soft tissues of the mouth, it is possible that a repellent material might offer little or no protection when coated upon, or impregnated in various types of packaging materials. In order to establish the ultimate utility of a repellent, it would be necessary to conduct additional tests designed to determine the most effective manner of application.

EXPERIMENTAL[1]

Experimental Animals.—The animals used in these studies were healthy, young rats, selected at random as to sex, but weighing a minimum of 150 Gm. Both albino and hooded laboratory rats were used.

Diets.—The diets used in these studies were of the type classified as a normal rat food. They were adequate for normal growth and reproduction, and were ground to pass a 10-mesh standard screen. Unless otherwise specified at the start of an experiment, the portions of treated food (i.e., food containing the candidate material) contained 2% by weight of the sample. Mixing was done by quartering and rolling, or by passing the diets through a small Wiley mill equipped with a 20-mesh screen.

* Received March 13, 1948, from the Fish and Wildlife Service, U. S. Department of the Interior, Patuxent Research Refuge, Laurel, Md.

[1] This procedure was adapted from that followed by Mr. J. F. Welch, Fish and Wildlife Service, Wildlife Research Laboratory, Denver, Colo.

Procedure.—Each experimental group consisted of three or more rats, housed in individual cages, and furnished water *ad libitum*. The rats had been maintained on the untreated experimental diets for a period of at least one week prior to the start of the test. At the beginning of the test, each animal was provided with a suitable food cup containing 20 Gm. of the untreated food, and a similar cup containing 20 Gm. of the treated food. No other food was available to the rats during the test period. The amount of food remaining in the cups was weighed each twenty-four hours during the four-day experimental period, and the cups returned to the cages. The animals, and any residual food, were discarded at the end of the experiment.[2]

DISCUSSION

One of the prime essentials in the evaluation of the results of these feeding tests was the establishment of some means for the expression of repellency. If all compounds were either completely accepted or completely rejected by the rat, the classification would be simple. However, the majority of the compounds tested fall somewhere in between these classifications, and it is desirable to be able to rate such compounds in terms of relative effectiveness. An index of repellency should permit direct comparison of the relative efficacy of materials tested at different times, and should be designed to account for a number of variables, such as the body weights of the experimental animals, the relative rates of acceptance of treated and untreated foods, the total amounts of food ingested during the test period, and the total consumption of the test material.

In the initial stages of the study, the test materials were evaluated solely on the basis of the relative acceptance of treated and untreated foods. It was assumed that any observed differences in the rates of acceptance could be attributed to the degree of repellency of the test material, and that all of the untreated food would be consumed before notable amounts of a repellent treated food would be eaten. In that case, it might be expected that all of the untreated food would be gone by the end of the second day, and that the animal then would have the choice between fasting or eating the treated food. As a final assumption, it was decided that in cases where 25% or less of the treated food was accepted during the experimental period, the test compound would be rated as a potential repellent, and would be subjected to further study.

This method of evaluating repellents was lacking in a number of respects. It did not allow for variations in the weights of the experimental animals, nor for differences in the animals' reaction to different types of compounds. Some compounds which produce physiological effects may be accepted initially, and then avoided. Other compounds may produce complete rejection of the treated food during the first two days, with slight acceptance during the remainder of the period when the hunger drive is greater. The first type might be worthless as a repellent, while the second might possess marked promise. As shown in Table I, the test scores fo two such dissimilar compounds might be identical i determined on the above basis.

Although these two compounds have the sam score on this basis, it is obvious that they should no be rated as equally effective repellents. The foo consumption for Compound A is typical for com pounds which possess no initial repellency, but pro duce some physiological reaction after ingestion The figures given for Compound B are typical fo materials which are truly repellent, in that the ani mals refused to accept any treated food unti motivated by extreme hunger.

In order to arrive at a more satisfactory metho for the interpretation of results, it is first necessar to define the desired pattern of repellent action. The figures given for Compound B may be taken as typical for a true repellent, in that all of the untreated food was consumed during the first two days of the test, while little or no treated food was consumed except under the drive of extreme hunger during the latter part of the experimental period. It remains, then, to develop a formula, or index of repellent action, which will account for such variables as physiological effects, random feeding, apparent attractiveness and variations in animal weights. In order to include these factors in a single index number, it is essential that the number include several parts. It should include two principal divisions, one each for the treated and untreated foods. Each division should be subdivided into four parts to represent the daily acceptance of the foods, and in order to have the expression of repellency in convenient numbers, 100 may be chosen as representing perfect repellency. The index number then would be:

$$K = 100 - (aT_1 + bT_2 + cT_3 + dT_4)(eU_1 + fU_2 + gU_3 + hU_4) \quad (1)$$

where $T_1 \ldots T_4$ represent the daily consumption of treated food; $U_1 \ldots U_4$ represent the daily consumption of untreated food, and $a \ldots h$ are weighting factors of undetermined magnitude. The product of the treated and untreated food divisions was chosen in order that the association of the two would be emphasized.

In order that the index number would reflect the desired pattern of repellent action, it was apparent that the weighting factors should be chosen so that a would have the highest value in the treated food division; b, c, and d would each be smaller than the preceding factor; while e and f must be smaller than g and h. Also, since the untreated food division should equal 1 in cases of normal acceptance, it should be divided by the total quantity of untreated food present (20 Gm.). Furthermore, since it is desired to have the index reflect the consumption in terms of milligrams per kilogram body weight, this figure should be divided by the body weight. For the purposes of simplicity of calculation, the average body weight was assumed to be 200 Gm. (0.200 Kg.) and since the values for K are relative, this will apply to any other body weight. The whole untreated food division then is divided by 100,000, since the total amount of food present (20,000 mg.) divided by the assumed body weight of the animal (0.200 Kg.) equals 100,000.

Calculation of the treated food division is more difficult. Here it is desired to express consumption

[2] Under normal conditions, rats of this weight should consume approximately 15 Gm. of food per day. Failure to ingest this amount of food may be attributed to abnormalities in the test animal, or to the effects of the candidate material in the treated food.

TABLE I.—EXAMPLES OF POSSIBLE SCORES ON THE BASIS OF FOOD CONSUMPTION

Compound	Animal Wt., Gm.	Type Bait	Daily Food Acceptance, Gm. 1 Day	2 Days	3 Days	4 Days	Score
A	150	*T*	2.0	0.0	0.0	0.0	2/20
		U	3.5	4.5	6.0	6.0	
B	275	*T*	0.0	0.0	1.0	1.0	2/20
		U	20	...	...	...	

in terms of milligrams per kilogram of the test compound. On the basis of the data from a number of tests, it was assumed that the average consumption of treated food was approximately 5 Gm., and since this food contained 2% of the test compound, this would be equal to 100 mg. of the repellent. If this value is introduced into the equation, it would be necessary to divide the weighting factors by 100 in order to bring the value of K to its former level. It is more convenient to use the original equation, and to include provision for the variation in body weights while expressing food consumption in grams:

$$K = 100 - (1/100W)(8T_1 + 4T_2 + 2T_3 + T_4)(U_1 + U_2 + 2U_3 + 4U_4) \quad (2)$$

where W is the body weight in kilograms.

Although this form of the equation proved satisfactory in a number of cases, it was found to be inadequate in cases of physiological repellency where a considerable fraction of the untreated food remained at the end of the experimental period. The low consumption of both treated and untreated food tended to give a relatively high score to compounds which could not be considered satisfactory repellents. To compensate for this, a fifth term (X), representing the amount of untreated food remaining at the end of the test period, was added to the untreated food division. In order to place proper emphasis on this term, it was assigned a weighting factor sufficiently high to produce a marked effect on the value of K. The final equation then became:

$$K = 100 - (1/100W)(8T_1 + 4T_2 + 2T_3 + T_4)(U_1 + U_2 + 2U_3 + 4U_4 + 8X) \quad (3)$$

This equation for the calculation of the degree of repellent action has been applied to the data from tests on some 1800 compounds. It has been found to furnish a rapid means for the evaluation of these data, and to allow ready comparison of the results obtained with different materials tested at different times. Examples of the results on compounds selected at random from our files are presented in Table II.

The data in Table II are typical of those obtained in the routine feeding tests. Inasmuch as relatively few animals are used in such tests, it might be questioned whether the data would be reproducible in subsequent trials. In order to determine this, and to establish the accuracy of the method, a series of tests were run on five compounds which appeared

TABLE II.—EXAMPLES OF K VALUES CALCULATED FROM EXPERIMENTAL DATA

Sample No.	Animal Weight, Kg.	Type Bait	Daily Food Consumption, Gm.[a] 1 Day	2 Days	3 Days	4 Days	K Value
701	0.335	T	0.3	0.4	2.3	1.7	94.5
		U	16.3	3.7	...	...	
702	0.268	T	0.0	0.0	1.0	0.0	98.5
		U	20	...	...	...	
703	0.237	T	0.7	0.3	1.7	0.3	86.5
		U	1.0	9.7	2.7	6.0	
704	0.185	T	0.0	0.0	2.0	0.0	95.7
		U	12.0	8.0	...	...	
705	0.191	T	0.0	0.0	0.7	0.7	97.9
		U	15.0	5.0	...	...	
706	0.165	T	0.7	0.3	2.5	1.5	78.7
		U	7.0	7.0	3.0	0.0	
707	0.186	T	0.0	0.0	0.3	0.0	99.4
		U	16.0	4.0	...	...	
708	0.155	T	0.3	5.7	2.0	3.7	59.1
		U	15.7	4.3	...	...	
709	0.167	T	2.7	3.0	1.0	4.0	−36.4
		U	7.0	4.7	2.3	1.7	
710	0.159	T	4.3	6.0	5.7	4.0	17.1
		U	11.7	5.0	3.3	...	
711	0.151	T	0.3	0.4	3.7	8.0	73.1
		U	12.3	7.7	...	...	
712	0.146	T	1.7	3.0	5.0	8.0	47.9
		U	12.3	7.0	0.7	...	
713	0.231	T	0.0	2.0	1.3	4.0	80.7
		U	11.7	4.3	0.7	1.6	
714	0.192	T	3.7	11.7	4.7	...	11.1
		U	15.0	5.0	...	...	
715	0.172	T	0.3	0.4	1.0	1.6	47.5
		U	3.7	0.6	0.7	1.7	

[a] ... indicates that all food had been consumed prior to weigh period.

to be active repellents. The resulting data are summarized in Table III.

As shown by the relatively low standard deviations from the means, the values for K are readily reproducible, and it is felt that this procedure furnishes a satisfactory procedure for the evaluation of the degrees of repellent activity shown by different chemical compounds. Through its use it has been found possible to assign numerical ratings to the 1800 compounds which have been examined, to obtain a measure of the relative efficacy of different classes of compounds and substituent groups, and to select those compounds and groups of compounds offering the greatest promise for further study.

TABLE III.—K VALUES OBTAINED IN A SERIES EXPERIMENTS ON SELECTED COMPOUNDS

Sample No.	No. of Observations	K Values Min.	Max.	Mean	Std. Deviati from Mean
1400	24	75.0	100	86.8	8.80
1414	27	78.7	100	95.1	6.99
1427	27	80.5	100	94.5	5.04
1434	22	72.0	97.5	92.5	5.00
1469	84	85.0	100	98.01	2.78

SUMMARY

A biological assay procedure and a method for the numerical expression of results have been devised for the determination of the repellency rodents of different chemical compounds. T procedure is based upon the degree of accep ability of foods containing the candidate rep lents, and has been shown to offer a rapid, reliab measure of repellent activity.

Certain Chemical Plant Growth Regulators and Alkaloid Formation in *Datura stramonium* L.*

By D. P. N. TSAO and H. W. YOUNGKEN, JR.

Datura stramonium L. plants were grown in nutrient solutions to which pyridine, piperidine, atropine sulfate, and 2,4-dichlorophenoxy acetic acid were added. Plants were grown to maturity and total alkaloid formation was measured. It is concluded that these growth-regulating substances in tolerated doses do not significantly alter the biosynthesis of *Datura* alkaloids.

IN PLANT growth treatments which make use of organic chemicals the problem arises as to what effect the growth regulator has upon the formation of alkaloidal constituents. Youngken and Fischer (1) have shown that pyridine, piperidine, and atropine sulfate will retard growth in *Datura* in large concentrations but each can be tolerated without growth effects in amounts less than 0.01 per cent (1 : 10,000). Ciamician and Ravenna (2) inoculated the above-ground portions of *Datura* and *Nicotiana* species with pyridine and piperidine and found that both substances disappeared in the plants; also that alkaloidal bases were obtained in greater yields. Pyridine produced the greatest increase. Youngken (3) described the toxic effects produced by 2,4-dichlorophenoxyacetic acid in *Datura stramonium* to show that this chemical substance is absorbed by the stem and that when so applied it had no significant effects upon total alkaloid formation in the leaves.

The extensive research of Dawson and his coworkers (4, 5, 6) clearly confirmed that the root of *Datura* and *Nicotiana* species is the sole seat of alkaloid synthesis in these plants. Thus, it would seem that organic chemical substances taken in through the underground portions would effect changes in alkaloid biosynthesis in most striking degree if at all. In view of these indications efforts were made to study the effects of pyridine, piperidine, atropine sulfate, and 2,4-dichlorophenoxyacetic acid when applied to the root and stem portions by continuous sand culture, ointment application, and inoculation methods.

EXPERIMENTAL

Normal diploid green stem *Datura stramonium* plants were grown from seed and transplanted to each of three experimental conditions, nutrient sand cultures, greenhouse soil plots, and the field. All plants selected for transplanting were as uniform in size and development as possible. Most had attained a height of from 8 to 10 inches with the first flower bud visible but unexpanded. The nutrient solution used in pure quartz sand was made accord-

* Received Aug. 11, 1948, from the College of Pharmacy Plant Laboratories, University of Washington, Seattle, Wash. Presented to the Scientific Section, A. PH. A., San Francisco meeting August, 1948.

ing to a modified Hoagland formula (1). Care was taken to maintain this solution in as nearly an acid state as possible so that the more favorable factor of an alkaline medium might not influence too greatly the results. (See McNair (7).) Nutrient solutions then ranged between *p*H 6.0 and *p*H 6.37. The soil in both plots, field and greenhouse, averaged *p*H 7.13. It consisted chiefly of 1 part humus and 2 parts sand. Sand cultures for root immersion were kept saturated with 0.001% (1 : 100,000) each of pyridine, piperidine, atropine sulfate, and 2,4-D in nutrient solution by flushing with fresh solutions once a week and maintaining a constant solution table of from 2 to 3 inches. The latter was effected by adding sufficient amounts of freshly prepared solution for each treatment during interim periods of each week. These treatments were begun early in May, 1947, and continued until August 8 of the same year. The acidity and alkalinity of the solutions were observed and are reported in Table I.

TABLE I.—*p*H AVERAGES[a] DATURA STRAMONIUM HOAGLAND NUTRIENT FORMULA WITH GROWTH REGULATOR ADDED (1)

	Per Cent in N. S.	Fresh Solutions, Average *p*H	Solutions Drained Once Weekly, Average *p*H
Pyridine	0.001	6.15	7.20
Piperidine	0.001	7.10	7.42
Atropine Sulfate	0.001	5.79	6.81
2,4-D	0.001	5.18	5.83
Control	...	6.31	7.36

[a] The averages are computed from weekly determinations made during nine weeks of continuous chemical application.

Soil plots were treated with the same percentage of each chemical. Plants grown in the greenhouse soil plots were treated by applying the substance incorporated in lanolin which was then smeared on the lower stem portion approximately one inch from the base. Two such applications were made, one during the first week following transplanting and the second at the end of forty-five days of growth. Such treatments were begun in March, 1947, and roots and leaves were collected on June 5.

Attempts were made to inject the chemicals into the stem lumen of plants grown in field plots with 5 cc. of each chemical in distilled water and then 2 cc. of the same. It was found that it was possible to insert a 26-gauge needle into the green stems of young ten- to twenty-day-old seedlings and that most of the solution could be injected. Injections were attempted twice weekly. However, as each plant matured this procedure became less satisfactory and following four weeks of continuous injections the method was discontinued. No abnormal morphological growth effects were observed. Leaves and root portions were collected at once and dried for alkaloid study.

As was expected from previous reports in the literature (1) plants treated with solutions of pyridine, piperidine, and atropine 0.001%, each in sand cultures, developed as vigorously as did the controls. It was interesting to note that following six weeks of continuous growth in such chemical media *Datura* plants receiving piperidine and atropine appeared stimulated slightly and most grew larger than did the controls (Fig. 2 and Fig. 3). (Note weight factors in Tables II and III.) Plants receiving 0.001% 2,4-D, on the other hand, showed toxic effects (Fig. 1) comparable to those reported previously by Youngken (3) when he used 0.1% to 5.0% ointment and applied it to stem parts. Leaves showed marked thickening, increased pubescence, and roughening. On the other hand, stems generally remained upright. Several spineless capsules developed. It

Fig. 1.—*Datura stramonium* L. (60 days). Left: Plant treated with 0.001% 2,4-D in sand culture. Right: Untreated control.

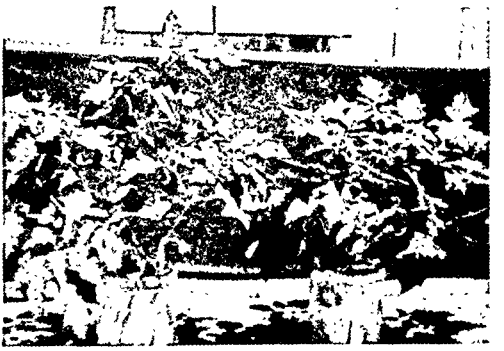

Fig. 2.—*Datura stramonium* L. (60 days). Left: Plant treated with 0.001% piperidine in sand culture. Right: Untreated control.

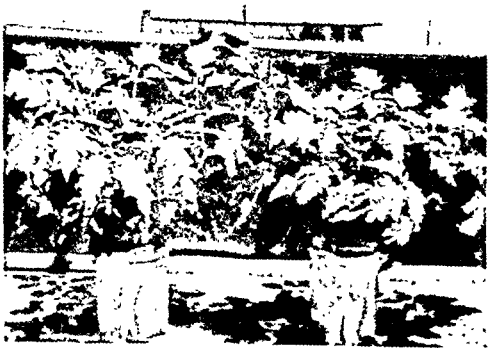

Fig. 3.—*Datura stramonium* L. (60 days). Left: Plant treated with 0.001% atropine sulfate in sand culture. Right: Untreated control.

TABLE II.—DATURA STRAMONIUM: PER CENT OF TOTAL ALKALOID FOLLOWING CHEMICAL TREATMENT IN SAND CULTURE: DRY WEIGHT CALCULATIONS

	Wt. Root Material, Gm.	Lot No.	Per Cent Total Alkaloid	Wt. Leaf Material, Gm.	Lot No.	Per Cent Total Alkaloid
Pyridine, 1:100,000	33.015	1	0.426	63.000	1	0.307
		2	0.418		2	0.329
		3	0.379		3	0.326
			Av. 0.408			Av. 0.320
			SD+[a] 0.019			SD+[a] 0.051
Piperidine, 1:100,000	42.501	1	0.381	76.400	1	0.287
		2	0.402		2	0.258
		3	0.317		3	0.289
			Av. 0.366			Av. 0.278
			SD− 0.023			SD+ 0.009
Atropine Sulfate, 1:100,000	42.210	1	0.391	75.346	1	0.299
		2	0.476		2	0.334
		3	0.431		3	0.331
			Av. 0.432			Av. 0.321
			SD+ 0.043			SD+ 0.052
2,4-Dichlorophenoxy Acetic Acid (2,4-D), 1:100,000	31.510	1	0.424	45.012	1	0.314
		2	0.372		2	0.344
		3	0.450		3	0.336
			Av. 0.415			Av. 0.331
			SD+ 0.026			SD+ 0.062
Controls	46.201	1	0.467	74.760	1	0.253
		2	0.298		2	0.282
		3	0.404		3	0.271
			Av. 0.389			Av. 0.269

[a] SD = Significant Difference

TABLE III.—DATURA STRAMONIUM: PER CENT TOTAL ALKALOIDS FOLLOWING USE OF NONTOXIC AMOUNTS 2,4-D, IN SAND CULTURE: DRY WEIGHT CALCULATIONS

	Wt. Root[a] Material, Gm.	Wt. Leaf Material, Gm.	Lot No.	Per Cent Alkaloid	
2,4-D, 0.001% (Toxic Check)	11.406	28.000	1	0.259	
			2	0.240	
			3	0.259	Av. 0.253
					SD− 0.012
2,4-D, 0.0001%	19.801	50.811	1	0.244	
			2	0.236	
			3	0.242	Av. 0.240
					SD− 0.025
2,4-D, 0.00001%	14.600	43.107	1	0.257	
			2	0.270	
			3	0.273	Av. 0.266
					SD+ 0.001
Controls	13.764	42.045	1	0.263	
			2	0.265	
			3	0.268	Av. 0.265
					SD+ 0.300

[a] Root material was not assayed for alkaloids because of insufficient material.

was, therefore, concluded that although 2,4-D when applied in ointment to the stem in the strength of 0.001% was nontoxic, it was absorbed by the root portions more completely in the same strength so that toxic manifestations then resulted. The leaves and roots of all plants treated during the summer of 1947 were collected, dried quickly at a temperature of 80° F., and set aside for alkaloid assay.

Due to the above toxic effects observed following the use of 2,4-D of 0.001% dilution in sand culture it was found necessary to reduce the strength of the chemical for root treatments so that an alkaloid study could be based upon a nontoxic effect as well as a toxic effect. Therefore, a fourth experiment was attempted beginning in October, 1947, during which plants were grown in cultures to which 0.001%, 0.0001%, and 0.00001% of 2,4-D in nutrient solution were added.[1] The toxic growth effects previously noted following treatments with 0.001% (1 : 100,000) were confirmed. Plants receiving lesser amounts of 2,4-D exhibited no toxic effects. In general these appeared stimulated and two plants

[1] Acknowledgment is made to Mr. Y. Gradalin, University of Washington, who assisted with this phase of the growth study.

surpassed growth of the best controls. Following three months of growth leaves and roots were collected and made ready for alkaloid analyses (Table III).

Alkaloid Determinations.—The root and leaf materials collected from each experimental condition as described above were dried at approximately 27° C., ground into a No. 40 powder, and assayed for total alkaloids according to the U. S. P. XIII method for Stramonium (8). In cases where there was insufficient material available the standard procedure was modified so that smaller weight samples could be analyzed. The findings of these assays are presented in Tables II, III, IV, and V.

sulfate, and 2,4-D. The fact that the first two heterocyclic compounds are structurally similar to portions of the hyoscyamine molecule yet do not take part significantly in its biosynthesis suggests that the *Datura* alkaloids are quite stable within the plant and might not be directly correlated with "precursor" molecules of such a nature. It appears also that none of these substances nor their conversion products were utilized to any large degree as nitrogen sources for alkaloid synthesis.

The evidence indicating that each substance was absorbed by root and leaf portions of the plant is largely based upon morphological growth changes that can be observed following toxic doses. None

TABLE IV.—DATURA STRAMONIUM: PER CENT TOTAL ALKALOIDS FOLLOWING OINTMENT APPLICATIONS TO STEM BASES: GROWTH IN SOIL PLOTS

	Wt. Root Material, Gm.	Lot No.		Per Cent Total Alkaloids	Material, Gm.	Lot No.		Per Cent Total Alkaloids
Pyridine, 1:100,000	40.1	1		0.363	69.1	1		0.355
		2		0.442		2		0.357
		3		0.420		3		0.367
			Av.	0.408			Av.	0.359
			SD	0.015			SD+	0.016
Piperidine, 1:100,000	48.3	1		0.491	73.0	1		0.337
		2		0.570		2		0.428
		3		0.483		3		0.393
			Av.	0.515			Av.	0.386
			SD+	0.122			SD+	0.043
Atropine Sulfate, 1:100,000	45.7	1		0.343	76.2	1		0.218
		2		0.388		2		0.315
		3		0.412		3		0.214
			Av.	0.381			Av.	0.249
			SD−	0.012			SD−	0.094
2,4-D, 1:100,000	44.6	1		0.311	70.4	1		0.305
		2		0.461		2		0.273
		3		0.377		3		0.319
			Av.	0.383			Av.	0.299
			SD−	0.010			SD−	0.044
Controls	48.1	1		0.380	73.1	1		0.264
		2		0.392		2		0.421
		3		0.406		3		0.343
			Av.	0.393			Av.	0.343
			SDI	0.300			SDI	0.300

From the data obtained it is evident that plants treated with piperidine and atropine sulfate grew most favorably. Treatment with pyridine and 2,4-D, on the other hand, resulted in lower amounts of dried root and leaf material for assay. Yet the total alkaloid yields obtained from this less favorable growth were not always lower than those obtained from better plants. Therefore, there seems to be little correlation between the nature of the plant growth and its ability to form alkaloids. For example, plants receiving piperidine in sand culture grew better than those receiving 2,4-D. The latter plants, however, yielded averagely more alkaloid.

DISCUSSION

The above findings indicate in general that there is no significant effect produced on alkaloid synthesis in *Datura stramonium* when the root and above-ground portions are exposed for long growth periods to nontoxic quantities of pyridine, piperidine, atropine

of the trace dilutions could satisfactorily be tested for in so minute a quantity.

The fact that neither toxic nor nontoxic quantities of 2,4-dichlorophenoxyacetic acid effect any appreciable change in total alkaloid yield further suggests the probability that neither this substance nor its conversion products have any appreciable precursor value in *Datura* alkaloid synthesis. This confirms the findings of Youngken (3) who earlier showed that no significant alkaloid change was effected when stems were treated with 2,4-D ointment. It might imply also that alkaloid synthesis in *Datura* is not associated with somatic cell growth changes for it was definite that toxic quantities of 2,4-D did alter cell growth markedly but at the same time had no unusual effect upon alkaloid formation. Unless shown otherwise by the use of other organic substances it would seem, then, that alkaloid synthesis in *Datura* is a mechanism more closely allied with genetical factors (9) and that while it can be influenced by nitrogen-supplying elements, especially late during the growth period (10), these must be of simple structure.

TABLE V.—DATURA STRAMONIUM: PER CENT TOTAL ALKALOIDS FOLLOWING INJECTIONS OF CHEMICALS INTO MEDULLA OF STEMS: GROWTH IN SOIL PLOTS

	Wt. Leaf Portions, Gm.	Lot No.	Per Cent Total Alkaloids		
Pyridine, 1:100,000	104	1	0.307		
		2	0.298		
		3	0.342		
		4	0.323	Av.	0.317
				SD−	0.077
Piperidine, 1:100,000	117	1	0.371		
		2	0.264		
		3	0.411		
		4	0.376	Av.	0.355
				SD−	0.039
Atropine Sulfate, 1:100,000	107	1	0.305		
		2	0.311		
		3	0.346		
		4	0.327	Av.	0.322
				SD−	0.072
2,4-D, 1:100,000	106	1	0.283		
		2	0.301		
		3	0.276		
		4	0.354	Av.	0.303
				SD−	0.091
Controls	110	1	0.311		
		2	0.425		
		3	0.379		
		4	0.361	Av.	0.394
				SD±	0.300

SUMMARY

A study of the effects of certain plant growth regulators on total alkaloid yields in roots and leaves of *Datura stramonium* has been made.

Contrary to the report of Ciamician, *et al.*, there appears to be no significant effect on the yield of total alkaloids following continuous applications of pyridine and piperidine. Further, it has been shown that atropine sulfate does not significantly influence total alkaloid yields when induced into the plant by several methods.

Finally, a similar study employing toxic and nontoxic quantities of 2,4-D has confirmed the findings previously reported by Youngken (3) and has shown that during toxic growth in the plant caused by this substance there is no significant altering of total alkaloid in the leaves.

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Polarographic Studies of Diethylstilbestrol*

L. E. BINGENHEIMER, JR., and JOHN E. CHRISTIAN

Studies on the polarographic properties of diethylstilbestrol were carried out, with the idea of developing a polarographic assay for this and other synthetic estrogens. Polarographic reduction of diethylstilbestrol was not found to occur under the conditions studied. Minute quantities of the estrogen suppress the oxygen maximum in a solution of potassium chloride, the degree of suppression being approximately proportional to the concentration of diethylstilbestrol within a narrow range. This property is the basis for a suggested assay of preparations of diethylstilbestrol.

ALTHOUGH the polarographic properties of the natural estrogens have been thoroughly investigated, little appears to have been done with the synthetic estrogens. Present assays of synthetic estrogens fall almost entirely into the classes of colorimetric, spectrophotometric, and biological analysis. Wessely and Wratil (1) have taken a step in the direction of polarographic analysis of stilbestrol and related compounds, but without encouraging results. The authors have undertaken a series of studies to devise a workable polarographic analysis applicable to this group of compounds.

EXPERIMENTAL

The instrument used in these experiments was the Sargent Model XX visible recording polarograph. An attempt was made to maintain constant conditions throughout the entire work. A column of 40 cm. of mercury was maintained in the standpipe of the dropping electrode. All solutions were allowed to come to room temperature, which was maintained within a range of 25 ± 1°. For the quantitative part of the work, the following control settings were used: sensitivity, 3; sensitivity vernier, 25; initial potential, 0; span potential, 3; damping, 3.

In the recording of oxygen maxima, which generally occurred at a potential of not over −0.6 volt, multiple determinations were recorded on a single chart by returning the bridge control to zero following the drop in current after each maximum.

The first experiments were to determine what type, if any, of polarographic behavior is exhibited by stilbestrol. Electrolysis of solutions containing stilbestrol in concentrations from 10^{-4} to 3×10^{-4} molar failed to give polarographic reduction waves when the supporting electrolyte was 0.1 *N* potassium chloride in approximately 30% aqueous ethanol, and again when the supporting electrolyte was potassium chloride in 0.1 *N* potassium hydroxide. No polarographic oxidation was observed when the same solutions were electrolyzed with polarity of the electrodes reversed.

Stilbestrol completely suppressed the oxygen maximum in 0.001 *N* solutions of potassium chloride in 10% aqueous ethanol and in 0.01 *N* potassium hydroxide. The latter solution appears to be more desirable, as ethanol has a slight suppressing action which interferes with the measurement of the activity of the stilbestrol.

For purposes of measurement, bubbling of oxygen, rather than air, through the solution was found to give much larger, more satisfactory maxima. These maxima were completely suppressed by as little as 10^{-5} molar stilbestrol, but almost no suppression was observed when the concentration was reduced to 10^{-6} molar.

As a basis for quantitative work the concentration of stilbestrol necessary to suppress the oxygen maximum by 50% was desired. This procedure was applied to alkaloids by Stackelberg and Schutz (2). A range of solutions from 0 to 10^{-5} molar in potassium chloride-potassium hydroxide supporting electrolyte were saturated by bubbling with oxygen for ten minutes. In all cases the degree of maximum suppression was approximately proportional to stilbestrol concentration from 0.2×10^{-5} to 0.8×10^{-5} molar, with approximately 0.4×10^{-5} molar required for 50% suppression.

Repeated electrolysis of solutions resulted in progressive decrease in the size of the maxima. This decrease is apparently irreversible. Further treatment of the solutions with oxygen did not restore the size of the maxima. A lowering of the *p*H of solutions after electrolysis was noted, but this effect probably is unrelated to the height of the maximum. When a supporting electrolyte corresponding to the buffer of Britton and Welford (3), containing potassium acid phosphate and sodium hydroxide (*p*H 11.45 at 25°), was used the tendency of the maxima to decrease in size was unchanged. The buffer exhibited a degree of maximum suppression which interfered with the measurement of stilbestrol.

Statistical evaluation of the results of repeated determinations of the same solutions showed that the per cent mean deviation in maximum size increases as the degree of suppression increases.

Tablets of stilbestrol were assayed polarographically by the maximum-suppression method. Extraction of the tablets with ether and sodium hydroxide test solution was carried out as described in the U. S. P. XIII assay for stilbestrol tablets, with the exception that an added extraction step through ether and base was found necessary to remove gelatin which interfered with the results. The residue after final evaporation of the ether was taken up in sodium hydroxide test solution. Potassium chloride was added and the solution diluted to correspond to 0.4×10^{-5} molar stilbestrol in 0.01 *N* potassium chloride and 0.001 *N* potassium hydroxide. Standards corresponding to the official limits of 90% and 110% of labeled strength were made up in a similar manner. When the solutions

* Received Sept. 27, 1948, from the School of Pharmacy, Purdue University, Lafayette, Ind.

were saturated with oxygen and electrolyzed, the size of the maximum in the unknown solution was between those of the standard solutions, indicating stilbestrol content within the desired range.

SUMMARY AND CONCLUSIONS

Diethylstilbestrol was found not to be polarographically reducible or oxidizable. It does, however, suppress the oxygen maximum, and this characteristic can be used for quantitative analysis. The degree of maximum suppression is roughly proportional to stilbestrol concentration in the range from 0.2×10^{-5} to 0.8×10^{-5} molar, with about 0.4×10^{-5} molar concentration required for 50% suppression of the maximum.

Repeated electrolysis of solutions results in decrease in maximum size, which is not restored on further treatment of the solutions with oxygen. Buffering does not prevent this action.

Quantitative determination of stilbestrol in pharmaceutical preparations by the degree of suppression of the oxygen maximum is possible. The main advantage of this method over the official assay is the time saving of about one hour. The main disadvantage of the method, especially in the assay of tablets, is the difficulty of removing gelatin, which interferes with the determination.

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Book Reviews

The Therapeutic Value of Penicillin. By DONALD G. ANDERSON and CHESTER S. KEEFER. J. W. Edwards, Ann Arbor, Mich., 1948. xiv + 297 pp. 15 x 24 cm. Price $7.00.

The Therapeutic Value of Streptomycin. By CHESTER S. KEEFER and WILLIAM L. HEWITT. J. W. Edwards, Ann Arbor, Mich., 1948. xiii + 289 pp. 15 x 24 cm. Price $7.00.

Long before penicillin and streptomycin were freely distributed, much was known about the clinical use of these drugs because of the study carried out by the Committee on Chemotherapeutics and other agencies of the National Research Council. These two reports are the summary and statistical findings of these committees; 10,000 cases in the instance of penicillin and 3000 in the instance of streptomycin are represented.

In each book, injections are broken down into convenient categories and the data are given in both tabular and discussion form. In addition, adequate attention is given to toxic and side reactions.

By using a mechanical sorting machine and by the tabulation of data on punch cards, the committee has been able to give details on apparent optimal dosage regimens, sex differences, degree of response with different complications and many other facts that would take years to accumulate in the usual manner. The amount of information to be found in these volumes of less than 300 pages is astounding.

The application of the contents of these books in a critical manner will prevent the abuse of two of the most valuable drugs the world has ever known. Nevertheless, precaution should be taken to avoid "freezing "progress under the assumption that these findings are entirely conclusive.

Bacterial and Mycotic Infections of Man. Edited by RENÉ J. DUBOS. J. B. Lippincott Co., Philadelphia, 1948. xiii + 785 pp. 17.5 x 25.5 cm. 101 illus. Price $5.00.

Under the influence of important changes in the collateral sciences of biochemistry, biophysics, physiology and pathology, bacteriology has been changing in its outlook during the past several years. A comparison of this book with past text and reference books in this field illustrates the change very forcefully. The reader will be impressed by the small amount of space devoted to morphology.

This book was written by a group of experts such as H. E. Alexander, B. D. Davis, H. Eagle, C. A. Janeway, H. R. Morgan, W. J. Nungester, A. B. Sabin, and A. M. Pappenheimer, Jr. Under the able editorship of René J. Dubos, a remarkable uniformity of style is present and there is no serious duplication of facts.

Chapters will be found on morphology, physiology, host-parasite relationships, the allergic state, immunology, blood groups, and the various bacteria and other microorganisms causing specific diseases. In addition there are chapters on principles of sterilization, principles of chemotherapy, and the cultivation and identification of pathogenic bacteria.

The National Foundation for Infantile Paralysis contributed financially to the preparation of this book and it is to be congratulated for sponsoring [illegible] worth while a project.

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The Stability of Thiamine Hydrochloride and Mononitrate in Parenteral Vitamin B Complex and Iron Solutions*

By ABRAHAM TAUB,† IRVING KATZ,‡ and MARTIN KATZ‡

Vitamin B_1, in parenteral solutions in admixture with riboflavin and niacinamide, exhibits maximum stability with respect to potency and clarity of solution at *p*H 4 under a nitrogen atmosphere. In admixture with iron compounds, vitamin B_1 is more stable in the presence of ferrous gluconate than of iron peptonate or ferric ammonium citrate. The optimum *p*H is 4. Of the specific grades studied, thiamine mononitrate is somewhat more stable than the hydrochloride in the above solutions.

ALTHOUGH thiamine hydrochloride in aqueous solution has been reported as stable below *p*H 5.5 (1), and capable of being sterilized at 120° for twenty minutes at *p*H 3.5 (2), evidence of minute decomposition in the form of discoloration and slight sedimentation, significant in parenteral solution manufacture, often becomes apparent within a few weeks to several months after preparation of Injection Thiamine Hydrochloride.

The exact mechanism of this decomposition, which is not correlated with any significant drop in potency, is not clearly understood. Oxidation leading to thiochrome formation seems to play only a subordinate role. Cleavage of the thiazole ring may give rise to the formation of colloidal solutions of sulfur and other degradation products, which under the influence of higher temperature during ampul sealing, sterilization or storage, or of catalysts in the form of trace metallic impurities in reagents employed, are coagulated to form visible particles or sediment. It has been estimated that decomposition of as little as 0.0001 per cent of thiamine can result in a noticeable precipitate (3).

The source of thiamine hydrochloride is an important factor in the preparation of a stable solution. Conformity to U. S. P. specifications is not in itself an indication of satisfactory quality for parenteral use. This has been recognized in a collaborative study in which there has been

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recommended a special ampul grade of thiamine (4). Such grades represent specially recrystallized batches of thiamine hydrochloride, empirically evaluated on the basis of satisfactory quality after several weeks' storage in solution. Although such grades have been commercially available, they are not the final answer to the problem. Ampul grades, though satisfactory when first obtained, frequently deteriorate slightly upon storage in the dry state after several months, even when kept below 20° under carbon dioxide. Ampul grades from the same sources of supply have been known to fluctuate markedly in quality indicating that the exact nature of the mechanism of induction of decomposition is not clearly understood.

The decomposition of thiamine injection is further aggravated when other B vitamins are added (5), or when certain iron compounds are introduced into the solution. Few published studies of these combinations appear, though they have been the subject of considerable empiric research as production problems in industry. That these studies have not met with marked success is indicated by the introduction of lyophilized B vitamin parenteral products to obviate the factor of deterioration due to the aqueous vehicle. Costliness of equipment and other factors have precluded the widespread adoption of this process.

EXPERIMENTAL

The introduction of thiamine mononitrate for experimental use in 1947, and preliminary studies pointing toward its greater stability than the hydrochloride (6), suggested the present study of comparative stability of the two salts in some commonly used vitamin B complex and iron solutions for parenteral use.

A comparison of some properties of both compounds is presented in Table I.

The acute oral and intravenous toxicity in mice and in rats is the same for both compounds (6, 7).

In solution at *p*H 4 the potency of the thiamine mononitrate has been reported as unchanged after more than twelve months; solutions in ampuls remain clear, with only slight discoloration, twenty-one months after autoclaving, if stored in the dark at room temperature (6).

In the present study the objectives were to determine the effect on stability of thiamine hydrochloride and nitrate solutions produced by (*a*) the presence of riboflavin and niacinamide at different *p*H levels and under an atmosphere of air or nitrogen; and (*b*) the presence of iron compounds, namely, the gluconate, peptonate, and green iron and ammonium citrate, at different *p*H levels.

A concentration of 50 mg. thiamine salt per cc. was used in all samples. The *p*H range was limited to 3–5. A lower *p*H gives rise to pain on injection; at higher *p*H, deterioration is rapid. In the B complex solutions, 2 mg. riboflavin per cc. was dissolved, using 200 mg. niacinamide per cc. to solubilize the riboflavin (8). Riboflavin and niacinamide were refluxed in solution for several minutes at boiling temperature, cooled to 25°, the thiamine salt added and the *p*H adjusted with hydrochloric acid or sodium hydroxide solution. Solutions after adjustment to desired volume were filtered aseptically through Selas 03 porcelain filter candles and filled into thoroughly washed clear glass Kimble ampuls, which had been dried in a dust-free atmosphere and sterilized. One-half of the lot was sealed under an atmosphere of purified nitrogen. Special care was exercised during filling and sealing to avoid formation of charred necks. Solutions in ampuls were sterilized at 100° for one hour. Although in commercial practice solutions containing thiamine are commonly sterilized by bacteriological filtration, in the present study the higher temperature was employed so that differences in stability between the thiamine nitrate and hydrochloride could be noted more speedily.

In the preparation of the thiamine and iron solutions the following variations from the above procedure were employed. Each solution contained in addition to 50 mg. of thiamine salt, 130 mg. of one of the iron compounds selected and 10 mg. of sodium citrate per cc., the latter to help keep the iron compound in solution. Solutions were prepared without heat. All ampuls were sealed under air and were sterilized at 15 lbs. for twenty minutes.

The following grades of chemicals were used: Thiamine Mononitrate, "for experimental use" (Merck); Thiamine Hydrochloride, ampul grade AA (Pfizer); Riboflavin U. S. P.; Niacinamide U. S. P.; Green Ferric Ammonium Citrate N. F.; Iron Peptonate N. F.; Ferrous Gluconate N. F.

All solutions in ampuls were tested for sterility, assayed by the thiochrome method, and inspected for clarity. Those free from suspended matter were stored in the dark, either at 45° for three weeks, or at room temperature (22° ± 5°) for one year.

The results are indicated in Tables II and III.

Although it was not the purpose of this study to determine the stability of the riboflavin in the above

TABLE I.—COMPARISON OF THIAMINE HYDROCHLORIDE AND NITRATE

	Thiamine Hydrochloride	Thiamine Nitrate
Formula	$C_{12}H_{17}ON_4SCl \cdot HCl$	$C_{12}H_{17}ON_4S \cdot NO_3$
Molecular weight	337.27	327.36
Potency	1 mg. = 333 I. U.	1 mg. = 343 I. U.
Description	White, crystalline powder with a slight odor	White, crystalline powder, nearly odorless
Moisture content (approx.)	4%	0.1%
Solubility in water	1 Gm./cc.	0.027 Gm./cc.[a]
Reaction of saturated solution	*p*H 2.5–3.0	*p*H 6.8–7.0

[a] At *p*H 4 the solubility is increased to 0.18 Gm./cc.

TABLE II.—RELATIVE STABILITY OF PARENTERAL SOLUTIONS CONTAINING THIAMINE HYDROCHLORIDE OR MONONITRATE IN THE PRESENCE OF RIBOFLAVIN AND NIACINAMIDE

pH	Thiamine Salt	Atmosphere in Ampul	Three Weeks at 45° % Thiamine Decomposition	Three Weeks at 45° Sediment[a]	One Year at 22° ± 5° % Thiamine Decomposition	One Year at 22° ± 5° Sediment[a]
3.0	Chloride	Air	25	4	5	4
3.0	Chloride	Nitrogen	24	4	5	3
3.0	Nitrate	Air	16	4	3	4
3.0	Nitrate	Nitrogen	14	4	2	2
4.0	Chloride	Air	23	3	8	3
4.0	Chloride	Nitrogen	23	3	6	3
4.0	Nitrate	Air	16	2	6	2
4.0	Nitrate	Nitrogen	15	2	4	1
5.0	Chloride	Air	30	2	21	2
5.0	Chloride	Nitrogen	27	2	15	2
5.0	Nitrate	Air	21	1	18	1
5.0	Nitrate	Nitrogen	17	1	12	0

[a] Degree of sedimentation is expressed numerically as follows: 0, clear; 1, very faint spiral cloud formed upon rotation of upright ampul; 2, slight cloud; 3, light sediment; 4, heavy sediment

TABLE III.—RELATIVE STABILITY OF PARENTERAL SOLUTIONS CONTAINING THIAMINE HYDROCHLORIDE OR MONONITRATE IN THE PRESENCE OF VARIOUS IRON COMPOUNDS

Iron Compound	Thiamine Salt	pH	Three Weeks at 45° % Thiamine Decomposition	Three Weeks at 45° Sediment[a]	One Year at 22° ± 5° % Thiamine Decomposition	One Year at 22° ± 5° Sediment[a]
Iron peptonate	Chloride	3.0	9	4	2	3
	Chloride	4.0	11	3	3	2
	Chloride	5.0	23	2	9	2
	Nitrate	3.0	9	2	2	2
	Nitrate	4.0	10	2	3	2
	Nitrate	5.0	14	1	10	1
Ferrous gluconate	Chloride	3.0	9	4	4	4
	Chloride	4.0	11	3	6	2
	Chloride	5.0	13	2	10	2
	Nitrate	3.0	11	3	4	3
	Nitrate	4.0	10	2	5	0
	Nitrate	5.0	13	2	10	0
Ferric ammonium citrate	Chloride	3.0	11	4	6	4
	Chloride	4.0	21	4	15	4
	Chloride	5.0	23	4	16	4
	Nitrate	3.0	10	4	4	4
	Nitrate	4.0	14	4	6	4
	Nitrate	5.0	15	4	9	4

[a] See footnote, Table II.

solutions, it is of interest to note that in a period of a year at room temperature the decomposition of riboflavin was found to be about 7% under air and 4% under nitrogen at pH 3, 9% under air and 7% under nitrogen at pH 4, and 24% under air and 18% under nitrogen at pH 5. No significant difference was noted between riboflavin decomposition in the presence of thiamine hydrochloride or mononitrate.

In addition to the degree of sediment indicated, a slight filming of the inner glass surface was noted in the iron peptonate and ferric ammonium citrate ampuls. The ferrous gluconate ampuls showed no filming at pH 4 and pH 5.

DISCUSSION AND SUMMARY

Thiamine decomposition in solution, after storage at 45° for three weeks, is more severe than after one year's storage at room temperature. Although the three weeks' test at 45° has been suggested as indicative of a year of shelf life for vitamin tablets and capsules, it is too rigorous an accelerated test for aqueous thiamine solutions.

The thiamine salts studied appear most stable, in the presence of riboflavin and niacinamide, at pH 3 with respect to potency, and are nearly as stable at pH 4. A pH of 5 is not a suitable environment. With respect to sedimentation the reverse is true; best results are obtained at pH 5 and nearly as good results at pH 4. An atmosphere of nitrogen yields slightly more stable solutions. The grade of thiamine mononitrate studied appears slightly superior to the grade of thiamine hydrochloride with respect to potency and clarity. It should be mentioned that preliminary tests of the grade of thiamine hydrochloride selected for this study showed the least amount of sedimentation upon prolonged storage

in aqueous solutions of all available grades at the time of the investigation.

Although optimum results are obtained by use of thiamine mononitrate under nitrogen at *p*H 4, complete clarity was not obtained after a year's storage at room temperature. It should be emphasized, however, that the parenteral solutions in this study were purposely sterilized at 100° or autoclaved to accelerate decomposition. It is reasonable to expect that if heat is avoided and solutions are prepared aseptically by sterile filtration, clarity over a longer storage period will be evident.

With respect to the iron compounds studied, thiamine salts show the poorest results in the presence of ferric ammonium citrate; marked sedimentation occurs under all conditions specified.

In the presence of either ferrous gluconate or iron peptonate, thiamine mononitrate shows little advantage over the hydrochloride with respect to potency or storage; both salts are more stable at *p*H 3–4 than at *p*H 5. However, the mononitrate shows lesser sedimentation than the hydrochloride. Both thiamine salts show less sedimentation at *p*H 5 than at *p*H 3. In the presence of ferrous gluconate, thiamine mononitrate at *p*H 4–5 yields solutions that remain clear after a year's storage at room temperature. At *p*H 4 the mononitrate in the presence of ferrous gluconate shows optimum stability both with respect to potency and clarity.

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Isolation of Oleanolic Acid and Ursolic Acid from *Thymus Vulgaris*, L.*

By EDWARD J. ROWE,† JACK E. ORR,‡ A. H. UHL, and LLOYD M. PARKS

Thymus vulgaris L. has been investigated for its nonvolatile constituents. Two previously unreported, isomeric triterpene hydroxy-acids were isolated. Oleanolic acid, $C_{30}H_{48}O_3$, was obtained from the petroleum ether extract and characterized through its acetate. Ursolic acid, $C_{30}H_{48}O_3$, was isolated from the ethyl ether extract and identified through its acetate, acetyl-acid chloride, and methyl ester.

THE CHEMISTRY of *Thymus vulgaris* Linné has, in the past, been largely restricted to the study of its volatile oil and constituents isolated therefrom. Comparatively little has been known of the nonvolatile constituents of the plant aside from the reported presence of salts, gum, resin (1), tannin (2), pentosan (3), and saponin (4). Therefore, further chemical investigation of the nonvolatile portion of this official drug seemed worth while.

In the course of this work, two previously unreported substances were isolated, one from the petroleum ether extract and the other from the ethyl ether extract. The physical appearance, solubility behavior, and high melting points of these substances suggested that they might be members of the polycyclic triterpenoid class of compounds. Proceeding on this assumption, the purified compound from the petroleum ether extract was identified through its acetate as oleanolic acid ($C_{30}H_{48}O_3$), a triterpene hydroxy-acid found in clove buds (caryophyllin), olive leaves, and many other plants. The purified compound from the ethyl ether extract was characterized through its acetate, acetyl-acid chloride, and methyl ester as ursolic acid ($C_{30}H_{48}O_3$), a triterpene hydroxy-acid isomeric with oleanolic acid and found commonly in *uva ursi*.

The only other plant in which the coexistence of these isomers has been reported is the grape

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(*Vitis vinifera* L.). Markley, Sando, and Hendricks (5) found oleanolic acid in the cuticle of the fruit, while Kuwada and Matsukawa (6) isolated ursolic acid from the cuticle of the leaves of the grape.

EXPERIMENTAL[1]

Isolation of Oleanolic Acid.—Eleven kilograms of finely ground, air-dried drug was extracted by continuous percolation with petroleum ether (Skellysolve "B") for about fifty hours, at which time a greenish substance began to settle out. The percolate was replaced with fresh solvent and extraction continued to exhaustion. The insoluble material, representing about 13% of the total petroleum ether extract, was dark green and readily pulverizable. Crystallization of the crude, green powder from alcohol yielded white, feathery, needle-like crystals. Several recrystallizations from alcohol produced a constant melting point at 307–308°. $[\alpha]_D^{22.6} = +78.85$ (20.8 mg. in 2 ml. $CHCl_3$). When mixed with a known sample of oleanolic acid (m. p. 307–308°), obtained from cloves by the method of Dodge (7), no depression in melting point was observed.

Dodge (7), Wedekind, *et al.* (8), and Markley, *et al.* (5), report a melting point of 310° while Winterstein (9) reports 306–308° for oleanolic acid. Van der Haar (10) reports $[\alpha]_D + 79.70$ ($CHCl_3$), and Wedekind (8) $[\alpha]_D + 79.6$ ($CHCl_3$) for oleanolic acid.

Acetate.—The acetyl derivative was prepared by gently refluxing 0.2 Gm. of the above with a mixture of 10 ml. of acetic anhydride and 0.5 Gm. of fused sodium acetate. The product, after crystallization from alcohol, melted at 262–263°. Mixture with a known sample of acetyl oleanolic acid (m. p. 262–264°) showed no depression of melting point. Dodge (7) reports a melting point of 260–265° and Markley, *et al.* (5) report 264.5–265.5° for the acetyl derivative.

Isolation of Ursolic Acid.—Following the petroleum ether extraction, the marc was exhaustively extracted with ethyl ether. A portion of the light green, readily pulverizable material obtained upon removal of the ether was dried, dissolved in alcohol, decolorized by heating under reflux for ten minutes with Norite, filtered, and cooled to room temperature, whereupon white, needle crystals melting at 278–281° were obtained Repeated crystallization from ethanol and methanol failed to produce any significant change in melting point. The entire ether extract was thus treated and about 80 Gm. of material of the above melting point was obtained.

The crystals were insoluble in water, 5% HCl, 5% NaOH, and moderately soluble in ether, ethanol, methanol, and chloroform at room temperature. The material dissolved in concentrated sulfuric acid producing a yellow solution. A potassium salt was prepared and found to be insoluble in ether and water, but soluble in alcohol.

Acetate.—The acetyl derivative was prepared by refluxing 10 Gm. of the above for eight and a half hours with a mixture of 50 ml. of acetic anhydride and 100 ml. of glacial acetic acid. The reaction product was crystallized from ethanol and recrystallized four times from methanol and once from acetone, yielding a final, constant melting point of 288–290°. A mixture with a known sample of acetyl ursolic acid (m. p. 290–291.5°) showed no depression of melting point. Sando (11) lists the melting point of acetyl ursolic acid as 289–290°.

Saponification of the Acetate.—The acetate was saponified by refluxing with methyl alcoholic potassium hydroxide for three hours. The reaction product was crystallized from methanol to a constant melting point of 282.5–284°. A mixture with a known sample of ursolic acid (m. p. 282–284°) showed no depression of melting point. A mixed melting point of the saponification product and the acetate showed a depression of 30–35°. Sando (11) and Jacobs (12) report a melting point of 284–285° for ursolic acid.

The optical rotation was determined for both the saponification product and a known sample of ursolic acid with these results: Saponification product $[\alpha]_D^{24.5} = +62.1$ (45.7 mg. in 2 ml. of 0.09 *N* methyl alcoholic KOH, α_D +1.42°, *l* 1 dm.). Ursolic acid—$[\alpha]_D^{24.5} = +62.6$ (40.6 mg. in 2 ml. of 0.09 *N* methyl alcoholic KOH, α_D + 1.27°, *l* 1 dm.).

Acetyl-Acid Chloride.—Two-tenths of a gram of the acetyl derivative was dissolved in 1 ml. of thionyl chloride, allowed to stand for two hours at room temperature, and then refluxed on a steam bath for half an hour. The excess thionyl chloride was distilled off under reduced pressure and the residue crystallized from a mixture of benzene and petroleum ether. Recrystallization from heptane and then from Skellysolve "B" gave white, needle crystals melting at 222°. A mixed melting point with a known sample of acetyl ursolyl chloride (m. p. 223°) showed no depression. Goodson (13) reports a melting point of 224–226° for this compound.

Methyl Ester.—The acid chloride was refluxed for six hours with methanol. The reaction mixture was then poured into water, the precipitate extracted with ether, and solvent removed by distillation. Crystallization of the product from methanol gave crystals melting first between 110–120°, then resolidifying, and finally melting sharply at 169°. After further crystallization only the 169° melting point was observed. Since this is the melting point of methyl ursolate, it was assumed that the acetyl group had been split off. This assumption was confirmed by a mixed melting point with known methyl ursolate (m. p. 170°) which showed no depression, and by refluxing known acetyl ursolyl chloride with methanol. The latter treatment yielded a product melting at 170° and which did not depress the melting point of the unknown.

SUMMARY

1. *Thymus vulgaris* Linné has been investigated regarding its nonvolatile constituents.

2. The substance isolated from the petroleum ether extract has been identified as oleanolic acid, a triterpene hydroxy-acid with molecular formula $C_{30}H_{48}O_3$.

3. The substance isolated from the ethyl ether extract has been identified as ursolic acid, a triterpene hydroxy-acid with molecular formula $C_{30}H_{48}O_3$ and isomeric with oleanolic acid.

[1] All melting points are corrected.

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A Toxicological Study of *p*-Dichlorobenzene*

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A determination of the LD_{50} dose of *p*-dichlorobenzene by the method of Bliss is presented. The results of a hematological study on three species of warm-blooded animals after a series of exposures to *p*-dichlorobenzene vapors indicate that this compound has a selective action on the granulocytes of the blood. Contrary to reports in the literature a histological study of twelve rabbits, thirteen rats, and eight guinea pigs, representing those animals dying or sacrificed after a series of exposures to *p*-dichlorobenzene vapors, revealed comparatively little liver damage or evidence of a definite hepatitis. Every animal, without exception, showed marked and extensive damage to the kidneys.

PARA-DICHLOROBENZENE or 1,4-dichlorobenzene, I. U. C.[1] is used extensively in the United States for insecticidal purposes and as a deodorant. In spite of its inception as a fumigant in 1914 (1), there is little information concerning its toxicity in the literature. Recently, however, PDB crystals were compared with DDT spray against fly larvae in outside pits, and proved to be more effective than the more highly publicized latter compound (2). The purpose of this paper is to contribute added toxicological information concerning this increasingly important compound.

The inhalation of PDB vapors in sufficient quantities over a long period of time may produce severe toxic effects in humans. Although the literature is comparatively barren of such reports, Perrin (3) reported the case of a 62-year-old man wearing clothes impregnated with PDB who developed asthenia and attacks of vertigo and whose blood picture showed anemia and hypogranulocytosis with normalization delayed for a long time. The picture was similar to that caused by benzene. Berliner (4) believed that in human subjects the toxic effects of PDB inhalation manifest themselves as hepatitis, loss of weight, and, later, cataract formation. Pike (5) discredited much of this work, especially with reference to the formation of a cataract. Although his rabbits showed, after repeated exposures to PDB vapors, some ocular pathology, he maintained that within seventeen days after the exposures had ceased all signs of eye pathology disappeared.

Ballotta and Mattioli (6) subjected rabbits to PDB vapors and claim that the blood picture showed only inconsiderable oscillation. No macroscopic or microscopic changes were perceptible in the various organs examined. They concluded that PDB vapors were harmless upon inhalation. Cameron, *et al.* (7), observed no significant changes in the organs of animals injected with PDB, but state that a slight liver necrosis may be produced. Teramoto (8) observed the effects of PDB vapors on guinea pigs, and noted that PDB caused only slight histological changes.

In view of the paucity of toxicological information concerning PDB and to the discrepancies observed in the literature, it appeared quite desirable to investigate further the toxic changes resulting from exposure to PDB.

EXPERIMENTAL

In all the ensuing experimental procedures pure PDB crystals "Mallinckrodt" (active ingredients 100%) were used.

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[1] Hereafter designated as PDB.

I. Determination of the LD_{50} Dose

According to Thienes (9) no definite information is at hand as to the acute toxic dose of PDB; therefore, a determination of the LD_{50} dose was undertaken. Eight groups of ten albino (Wistar strain) rats, 50% male and 50% female, weighing between 125 to 275 Gm., maintained at a temperature 22 ± 1° and fasted overnight, were injected intraperitoneally with a solution of PDB in peanut oil (200 mg. per cc.), and observed for a 96-hour period. Doses ranged from 2000 to 3400 mg./Kg The results are shown in Table I. The median lethal dose (LD_{50}), calculated by the method by Bliss (10), was found to be 2.562 ± S.E. 0.0125 Gm./Kg.

TABLE I.—RESULTS OF INTRAPERITONEAL INJECTIONS OF *p*-DICHLOROBENZENE IN PEANUT OIL INTO ADULT ALBINO RATS (WISTAR STRAIN)

Dose, Mg./Kg.	—18 Hours— Mortality	%	—24 Hours— Mortality	%	—36 Hours— Mortality	%[a]
2000	2/10	20	2/10	20	2/10	20
2200	3/10	30	3/10	30	4/10	40
2400	2/10	20	2/10	20	4/10	40
2600	2/10	20	3/10	30	4/10	40
2800	5/10	50	5/10	50	5/10	50
3000	6/10	60	6/10	60	6/10	60
3200	7/10	70	7/10	70	7/10	70
3400	5/10	50	5/10	50	7/10	70

[a] 48-, 72-, and 96-hour readings were the same as that recorded for 36 hours.

II. The Hematology of Warm-Blooded Animals Subjected to PDB Vapor Inhalation

A large chamber (102 x 45 x 46 cm.) with a capacity of 217.43 liters of air was constructed of one-quarter inch plywood. The interior was partitioned into three compartments by means of wire mesh and wooden slats. A hot plate with a piece of flat sheet metal on it was installed in the center compartment, the outer compartments being used for animals.

A calculated amount of PDB (21.7 Gm. equivalent to 100 mg. per liter of air) was weighed out, placed on the piece of sheet metal over the hot plate, and allowed to vaporize. Prior to this the animals were placed in the outer compartments and the chamber closed via a hinged outer door. The chamber was made further airtight by sealing with adhesive tape. The animals were exposed to the PDB vapors for a specified time in the chamber daily. An additional three minutes was allowed for the PDB crystals to vaporize. The temperature of the chamber was maintained at about 39–42°.

Three species of animals were used: rabbits, rats, and guinea pigs.

A. Rabbits.—A group of 18 male rabbits was used. The normal blood picture (white blood cell, red blood cell, and differential counts) and weight of each rabbit were determined before the first exposure. An additional rabbit was used as a control, making a total of 19 rabbits. All the rabbits, except the control, were exposed to PDB vapors in the chamber for a period of thirty minutes daily at an approximate concentration of 100 mg. PDB/liter of air. The control rabbit was placed in the chamber for the same period daily but without PDB vapors. At intervals of five to nine days the blood picture of each rabbit was noted, the marginal ear vein being the site of withdrawal of blood. An individual chart was kept for each animal and changes in weight and blood picture noted at intervals.

Resulting symptoms of exposure ranged from simple eye and nose irritation to the more usually encountered syndrome, viz., intense irritation of the eyes and nares, a pronounced "mark time" reflex, muscle twitches, tremors, loss of righting reflex, definite horizontal or vertical nystagmus, rapid but labored breathing. Recovery from symptoms ensued in from thirty minutes to two hours, depending upon the physiological state of the animal. The use of the hind legs was always the last function returning to normalcy.

A sample of the type of chart maintained is presented below (see Rabbit No. 1).

There was evidence of a granulocytopenia. Exposures ceased after the thirty-first day; blood picture was followed for return to normalcy. Twenty-seven days for normalization were needed.

A summarization of the effects of PDB vapors on rabbits after a series of exposures is given in Table II.

As seen in the Table, 11 rabbits exhibited a definite granulocytopenia, 3 were questionable, and 3 showed no decrease in the granulocytes (excluding the control). The weight decreased in 14 animals while only 3 showed an increase. An increase in total WBC's and RBC's was seen in 5 animals and a decrease in 12. The granulocytopenia and weight decrease can be considered as significant while the changes in total WBC and RBC counts are comparatively insignificant.

The 5 rabbits which were followed up after exposures ceased showed a normalization on the blood picture within thirty days.

RABBIT NO. 1

No. of Exposure	Normal	8th Day	15th Day	20th Day	29th Day
Differential, %					
Neutrophils	29	31	13	12	12
Eosinophils	1	0	0	2	3
Basophils	0	0	0	0	0
Myelocytes	3	0	1	1	2
Lymphocytes	62	66	84	82	79
Monocytes	4	3	2	3	4
Unknown	1	0	0	0	0
White Blood Cell Count	6,850	8,300	5,100	6,600	5,250
Red Blood Cell Count	5,350,000	5,450,000	5,610,000	5,260,000	4,970,000
Weight, Kg.	1.87	1.89	1.81	1.73	1.59

B. Rats.—A group of 20 albino (Wistar strain) rats, 50% male and 50% female, ranging in weight from 150 to 300 Gm., maintained at a temperature of 22 ± 1° and fasted overnight, was used in this experiment; 18 were subjected to PDB vapors for a period of twenty minutes daily while 2 were used as controls.

The resultant effects of exposure to PDB vapors shown by the rats were remarkably similar to those shown by the rabbits. After each exposure there was complete narcosis with attendant tremors and twitches of the extremities. This condition usually lasted from one-half to three hours.

As with the rabbits, individual charts were maintained for each rat and changes in the blood picture carefully noted. A summarization of the effects of PDB vapors on rats after a series of exposures is presented in Table III.

Eight rats exhibited a definite granulocytopenia, 3 were questionable, and 2 showed no evidence of a lowering of granulocytes. Nine rats showed a decrease in total WBC's while 4 showed an increase. The granulocytopenia and the tendency toward this condition seem to be quite significant.

C. Guinea Pigs.—The same experimental conditions as described for the rabbits and rats were employed. A group of 9 male guinea pigs was used in this experiment and subjected to PDB vapors for twenty minutes daily.

Only differential cell counts were done since the total white blood cell and red blood cell counts showed no great variation in previous experiments with other warm-blooded animals.

The guinea pigs exhibited much the same symptoms as did the rabbits and rats, the symptoms lasting about one-half to one and one-half hours. As with the rabbits and rats, individual charts were maintained for each guinea pig and hematological and weight changes carefully noted.

Repeated exposures to this concentration (100 mg. of PDB per liter of air) produced such a marked intoxication in the guinea pigs that many of them died after only comparatively few exposures. A summarization of the effects of PDB vapors on guinea pigs is presented in Table IV.

Five of the guinea pigs exhibited granulocytopenia, 2 showed a tendency toward lowering, while 2 showed no decrease in granulocytes. Six of the animals showed a weight decrease while 3 showed none. The tendency toward granulocytopenia in this group of animals is apparent and significant. Two-thirds of the animals showed a decrease in weight. The guinea pig does not tolerate PDB vapors well since only 1 of the 9 test animals survived twenty exposures.

III. Histological Study

Thirteen rabbits, 15 rats, and 8 guinea pigs used in the hematological study previously described, and representing those animals dying or sacrificed after a series of exposures to PDB vapors, were autopsied and gross and microscopical changes in certain organs noted. The lungs, liver, kidney, and heart were studied in greatest detail. Each of these organs was carefully examined for gross pathology, and a section of tissue was taken from each to be used for study of microscopic changes.

The following procedure was used for making and staining the sections.

A 5-mm. piece of the tissue was placed in Bouin's solution and allowed to remain there overnight. The following day the tissue underwent a dehydration process through treatment with the following mixtures for the period of time specified:

1. 75% ethyl alcohol and 25% water for two hours.
2. 60% ethyl alcohol and 40% water for six hours.
3. 50% ethyl alcohol, 20% water, 30% *n*-butyl alcohol for six hours.
4. 50% ethyl alcohol, 15% water, 35% *n*-butyl alcohol for six hours.
5. 35% ethyl alcohol, 5% water, 60% *n*-butyl alcohol for six hours.
6. 30% ethyl alcohol, 70% *n*-butyl alcohol for six hours.
7. 100% *n*-butyl alcohol for six hours (first change).
8. 100% *n*-butyl alcohol for six hours (second change).

TABLE II.—A SUMMARIZATION OF THE EFFECTS OF *p*-DICHLOROBENZENE VAPORS ON RABBITS

Rabbit No.	% Change in Granulocytes	No. of Exposures	Death or Follow-up	Change in Weight, Kg.	WBC Total	RBC Total
1	33–17	31	Follow-up	1.87–1.59	Decrease	Decrease
2	31–19	32	Follow-up	1.78–1.56	Increase	Increase
3	29–25	33	Death	2.53–2.27	Decrease	Decrease
4	28–19	33	Death	2.55–2.51	Decrease	Decrease
5	41–29	15	Death	1.60–1.71	Decrease	Increase
6	29–18	16	Death	2.20–1.74	Increase	Decrease
7	41–18	26	Death	1.69–1.39	Decrease	Decrease
8	36–33	29	Death	1.73–1.74	Decrease	Decrease
9	28–25	16	Death	2.10–2.08	Increase	Decrease
10	38–29	11	Death	2.12–2.19	Decrease	Increase
12[a]	43–29	12	Death	1.83–1.67	Decrease	Decrease
13	40–20	31	Follow-up	3.07–2.61	Decrease	Decrease
14	44–22	30	Death	2.33–1.85	Decrease	Decrease
15	31–20	31	Death	2.93–2.92	Decrease	Decrease
16	42–28	32	Follow-up	3.58–3.14	Increase	Increase
17	52–17	33	Follow-up	4.55–4.09	Decrease	Decrease
18	60–29	34	Death	3.41–2.93	Increase	Increase
19	34–35	30	Control	2.78–3.29	Increase	Decrease

[a] Rabbit 11 died after the first exposure and is omitted.

TABLE III.—A SUMMARIZATION OF THE EFFECTS OF *p*-DICHLOROBENZENE VAPORS ON RATS

Rat No.	% Change in Granulocytes	No. of Exposures	WBC Total	RBC Total
1	26–14	25	Decrease	Decrease
2	32–17	25	Decrease	Increase
3	22–18	25	Increase	Increase
4	36–37	25	Increase	Increase
5	40–33	11	Decrease	Increase
6	21–11	22	Decrease	Increase
7	36–16	22	Increase	Increase
8	34–17	22	Decrease	Decrease
9	37–16	22	Decrease	Decrease
10	31–18	22	Decrease	Decrease
11[a]	19–33	22	Decrease	Increase
15[b]	20–11	10	Decrease	Decrease
18[c]	17–14	18	Increase	Increase

[a,b] Nos. 12, 13, 14, 16, and 17 died after a few exposures and are therefore eliminated from the above table.
[c] Nos. 19 and 20 (controls) showed little deviation from normal.

TABLE IV.—A SUMMARIZATION OF THE EFFECTS OF *p*-DICHLOROBENZENE VAPORS ON GUINEA PIGS

Guinea Pig No.	% Change in Granulocytes	No. of Exposures	Change in Weight, Kg.
1	36–29	8	0.625–0.618
2	31–20	16	0.555–0.532
3	46–29	17	0.675–0.661
4	39–26	14	0.590–0.582
5	36–25	12	0.690–0.678
6	29–30	6	0.660–0.662
7	29–32	15	0.605–0.619
8	35–21	20	0.555–0.530
9	43–37	6	0.800–0.802

After dehydration the tissue was imbedded in "mush paraffin," a mixture of 20% *n*-butyl alcohol and 80% paraffin (Bioloid, m. p. 53–55°).

The tissue remained imbedded for a minimum of twelve hours, after which time transfer to pure paraffin was effected. After twelve hours in the pure paraffin, the tissue was ready for blocking.

A porcelain or glass boat of a suitable size was coated liberally with glycerin and pure melted paraffin poured into the container. The tissue was transferred into this container and the paraffin allowed to cool and harden. After complete hardening the block was loosened from the container and cut into rectangular or square sections to be fitted onto the pivot of the microtome.

A small amount of wax was built up on the pivot, heated slightly, and the section of imbedded tissue pressed upon the pivot which was then inserted into the microtome, ready for cutting. All sections were cut at a 10-μ thickness. A warm stage maintained at about 40° was brought into use. Glass slides with a drop of distilled water on each were placed on this stage and the cut sections laid flattened out on the slides. This greatly facilitated adherence of the cut section to the glass slide. The cut sections were allowed to remain on the warm stage overnight to insure further adhesiveness to the slide. The following morning the sections were ready for staining.

The slides were placed back to back in a staining dish carrier and immersed into each of the following solutions for a specified time:

1. Xylol—two to three minutes to dissolve the paraffin.
2. Xylol—two to three minutes, second change.
3. *n*-Butyl alcohol—two minutes.
4. Ethyl alcohol—two minutes.
5. 80% alcohol—two minutes.
6. Distilled water rinse—one minute.
7. Hematoxylin stain—twenty minutes.
8. Rinse thoroughly in tap water, removing all stain excess.
9. Immerse in acid-alcohol, with care, to decolorize.
10. Rinse thoroughly in tap water.
11. Eosin stain—three to five minutes.
12. Rinse well in tap water.
13. 70% alcohol—two minutes.
14. Ethyl alcohol—two minutes.
15. *n*-Butyl alcohol—one minute, first change.
16. *n*-Butyl alcohol—one minute, second change.
17. Xylol—two minutes, first change.
18. Xylol—until ready for mounting.

The slides were removed from the xylol and mounts made with either Clarite or Demar and covered with a cover glass. The slides were then allowed to dry overnight on the warm stage, being ready for microscopic examination the following morning.

Several sections of each organ from each animal were made and all deviations from the normal histological appearance carefully noted. The rabbits showed varying pathological changes and are therefore reported individually. However, the rats and guinea pigs as a group showed identical changes and consequently are reported as a group only.

A. Rabbits.—Twelve rabbits that died of exposure to PDB vapors during the hematological study previously described and one control rabbit were autopsied and sectioned. A typical report of gross and microscopic findings is presented. The results for Rabbit No. 3 are as follows:

Gross Pathology.—The left lung showed hemorrhagic areas; other parts of the lung appeared normal. The lungs were colored a dark red. The liver and heart appeared to be normal. The kidneys showed no visible changes except for a slightly swollen appearance.

Microscopic Pathology.—The lungs were markedly hyperemic and edematous with fresh hemorrhages in the bronchi and alveoli and large amounts of serous exudate in the bronchi. (See Fig. 1.) Atelectasis was very prominent along with occasional emphysemic areas. The microscopic findings preclude true pneumonia as the possible cause of death. The lymphoid tissue showed no hypertrophy; nuclei showed shrinkage and condensation (pyknosis).

The kidneys showed evidence of much damage characterized by the swollen epithelium of the uriniferous tubules, especially of the proximal and distal convoluted tubules. Epithelium of the tubules showed early albuminous degenerative changes. (See Fig. 2.) The nuclei of the cells showed loss of alignment and were arranged in a disorderly fashion. Many cellular casts were present in the collecting tubules. (See Fig. 3.) The glomeruli exhibited a definite hyperemia.

There was no apparent damage to the liver cells themselves. Many edematous and hyperemic areas

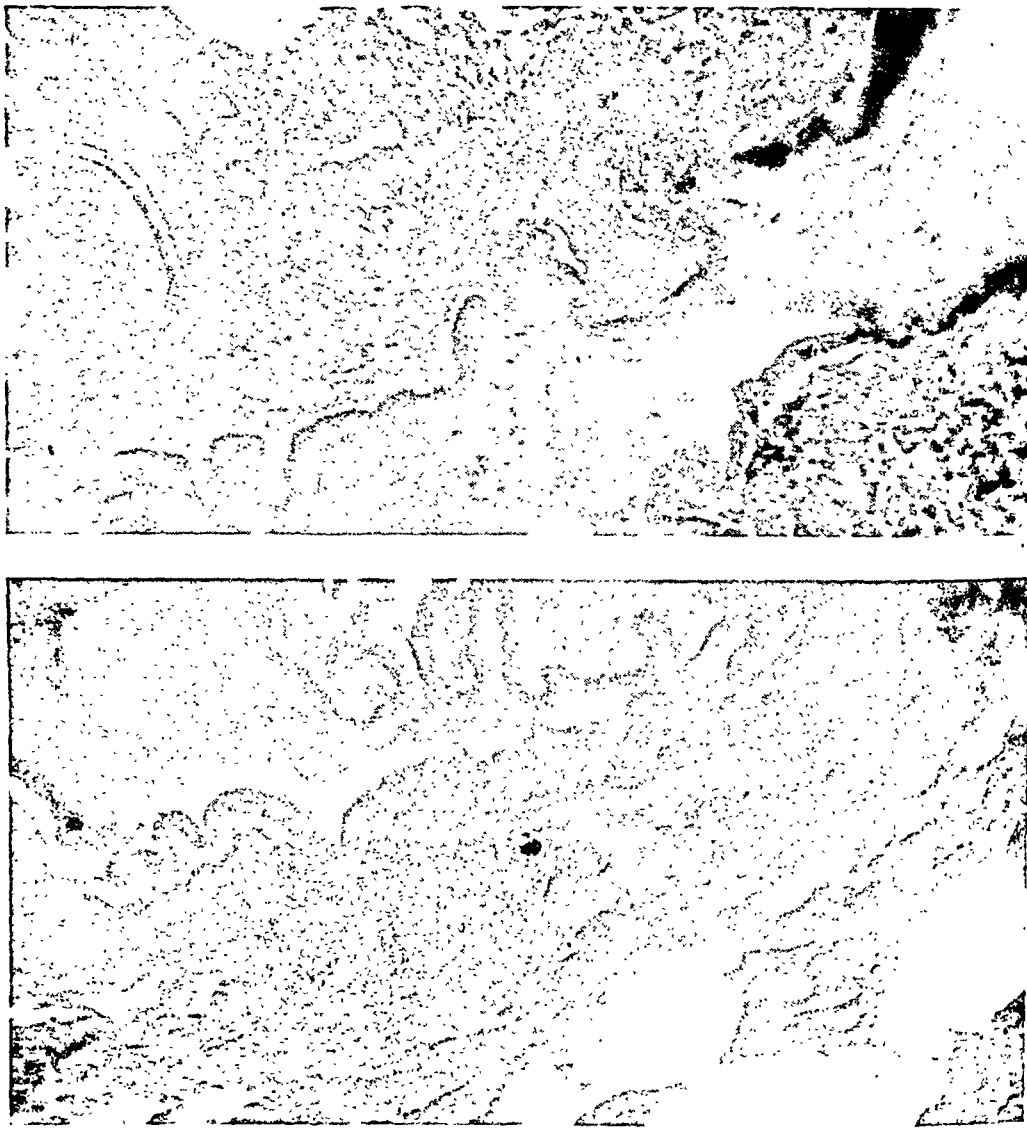

Fig. 1.—Photomicrographs showing serous exudate in bronchi of lung and loosening of the epithelium (rabbit).

NOTE: Many sections showed accumulations of serous fluid in the alveoli (rabbit, guinea pig, and rat).

were seen with an occasional lobule congested and the adjoining sinusoid space filled with blood. There was no evidence to indicate a definite hepatitis.

The heart showed no obvious damage. The nuclei were intact, there was some separation of muscle fiber, and occasionally a possible degenerative change in the muscle noted by the lack of striations in the muscle. However, the latter possibly could be due to fixing technique.

B. Rats.—Thirteen rats, sacrificed after a series of exposures to PDB vapors during the hematological study previously described, and two control rats were autopsied and sectioned. All the rats showed somewhat the same type of gross and microscopic pathology and are therefore presented as a group in the following report:

Gross Pathology.—All the rats, except the controls which appeared to be normal, exhibited hemorrhagic areas in the lungs of varying degrees as well as a very bright reddish color to either one or both lungs. The heart was of normal size and color in all animals. The majority showed no liver abnormality with the exception of a few which exhibited white streaks on the surface. The kidneys appeared to be normal except for a few that were swollen.

Microscopic Pathology.—The animals showed little variation of histological change. Hyperemic and edematous areas were most frequently encountered

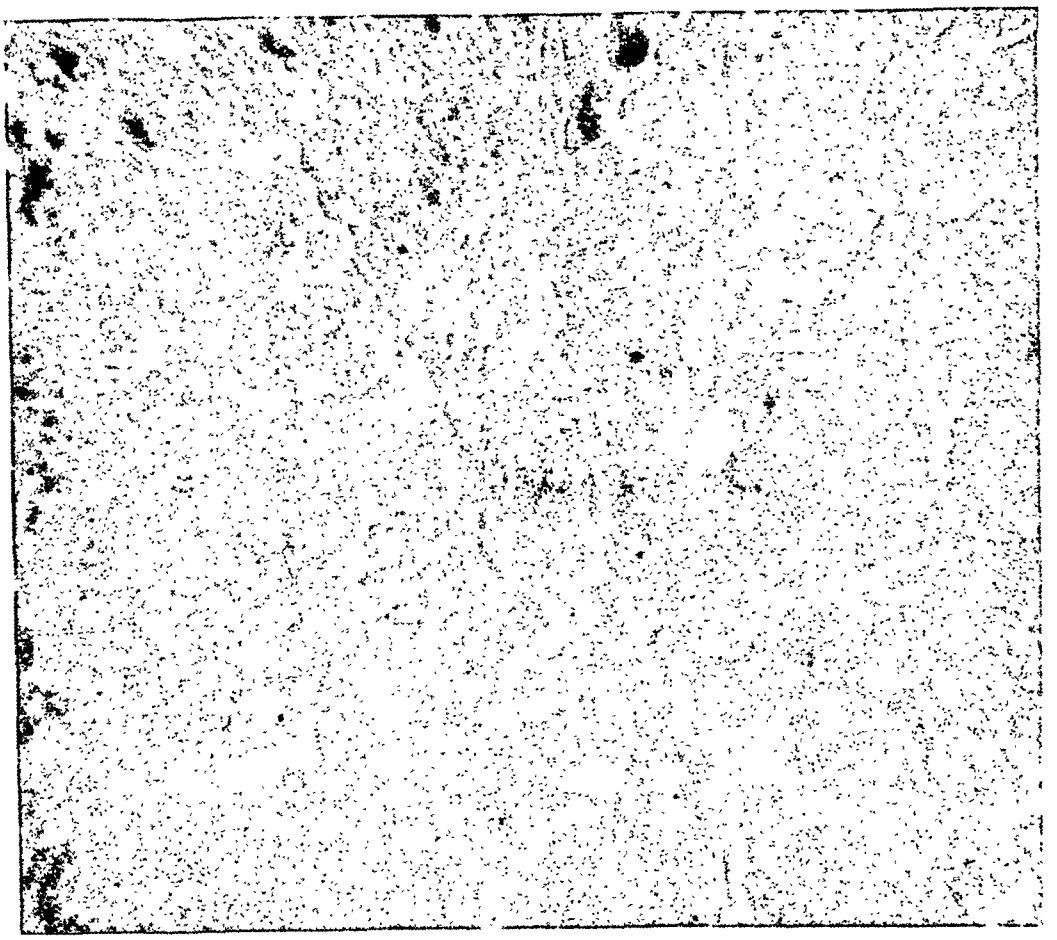

Fig. 2.—A photomicrograph of a rabbit kidney showing marked degenerative changes in the epithelium of the convoluted tubules. The glomeruli exhibit swelling of the tufts and pyknosis of the cells in the tufts.

in the lungs. The epithelium of the bronchi did not appear to be much changed in most cases. Occasionally a dissociation of the epithelium of the bronchi was seen but never very clearly. Fresh hemorrhages in the lungs were observed, mainly in the bronchi. No hypertrophy of the lymphoid tissue was noted. A pyknotic appearance of the nuclei was common. Neither damage to the cells nor a hepatitis was observed in the liver sections of the rats. Some hyperemic and edematous areas were seen. All the rats showed extensive damage to the kidneys. The epithelium of the tubules was swollen in most cases. The glomeruli were swollen and hyperemic with very marked pyknosis. Almost all the tubules showed varying degrees of necrobiosis. Some animals showed degeneration and loss of alignment of the nuclei. A few of the animals showed a granular exudate in the collecting tubules. Generally the damage to the kidneys was severe. No definite heart damage was observed. Occasionally an edematous area was noted but the nuclei and striation were intact and visible.

C. Guinea Pigs.—Eight guinea pigs were autopsied and sectioned and are presented as a group since the findings were not too variable.

Gross Pathology.—The lungs exhibited very dark hemorrhagic areas although the color of the lungs did not vary greatly from the normal. The kidneys did not appear to be swollen, but showed reddish dotted areas on the surface. Many of the animals had a white streak on the liver surface. The heart showed no visible changes.

Microscopic Pathology.—The lungs were, in general, markedly congested and edematous. Distention of the alveoli was a prominent feature. Dissociation and loosening of the epithelium of the bronchi were prominently observed. Emphysematous areas were encountered frequently. Some bronchi were filled with serous exudate and with blood from fresh hemorrhages. All the animals showed extensive kidney damage in the form of swollen as well as degenerated epithelium of the uriniferous tubules. The glomeruli were swollen and congested and pyknosis was much in evidence. No evidence of a definite hepatitis or cellular damage to the liver was observed. A few edematous and hyperemic areas were seen in the liver of some animals. The nuclei of the heart were intact. Occasionally an animal would exhibit some early changes in the form of an infiltration which was

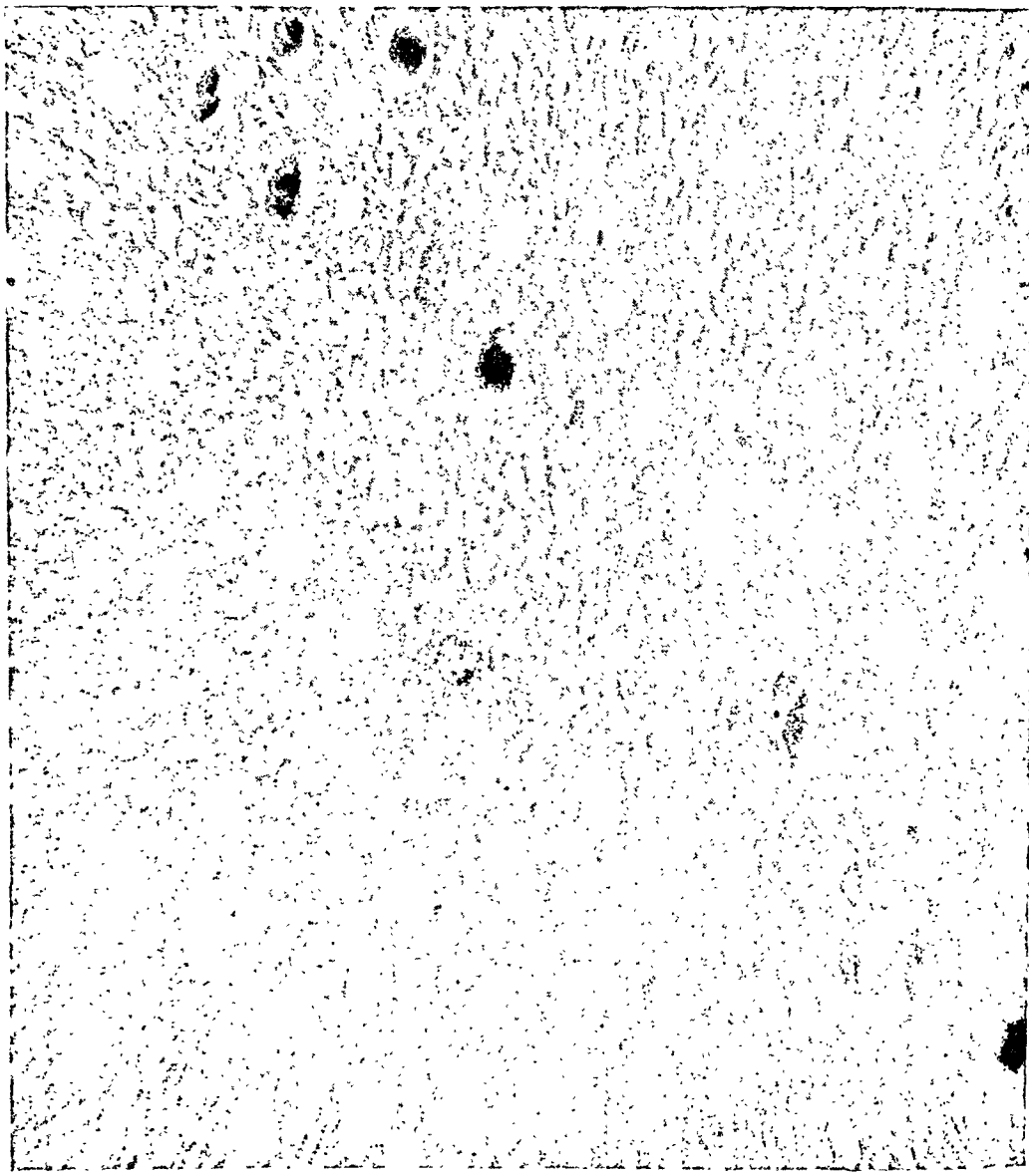

Fig. 3.—A photomicrograph of cellular casts in straight collecting tubules of rabbit kidney.

characterized by round cells—histiocytes and monocytes—indicating a possible myocarditis. In general, however, little or no pathology of the heart tissue was encountered.

SUMMARY

1. A determination of the LD_{50} dose of *p*-dichlorobenzene in peanut oil, via intraperitoneal injection, using albino (Wistar strain) rats has been presented. According to Bliss, this was found to be 2.562 ± S. E. 0.0125 Gm./Kg.

2. Inhalation of PDB vapors in sufficient concentration produced a marked intoxication in the rabbit, rat, and guinea pig characterized by a definite and constant syndrome. This syndrome consists of intense eye and nares irritation, tremors and twitches of the extremities, a "mark time" reflex, a loss of the righting reflex, a definite nystagmus, and rapid but labored respiration. Death may occur; if not, complete recovery ensues within one-half to two hours depending upon the physiological state of the animal.

3. The results of a hematological study of rabbits, rats, and guinea pigs after a series of exposures to PDB vapors indicate that this compound has a selective action on the granulocytes

of the blood, producing a granulocytopenia in a majority of the animals. The total leucocytic (showing some increase in lymphocytes) and erythrocytic counts were not greatly affected. Normalization of the blood picture occurred within thirty days after exposures were discontinued.

4. A histological study of twelve rabbits, thirteen rats, eight guinea pigs, representing those animals dying or sacrificed after a series of exposures to PDB vapors, revealed that PDB has a definite selective action on the kidneys. Every animal showed marked and extensive damage. Contrary to reports in the literature, comparatively little liver damage or evidence of a definite hepatitis was observed. There was the expected amount of damage to the lung tissue. Damage to the heart tissue was negligible.

5. Inhalation of PDB vapors produced severe toxic effects in small laboratory animals only if the concentration was unusually high and the vapors were inhaled over a sufficiently long period of time.

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Standard Tolerances for Pharmaceutical Compounding. A Basis for Their Establishment. II. Liquid Preparations*

BY SAMUEL W. GOLDSTEIN†, ‡, §

The validity of the method described in this report for the determination of reasonable and equitable standards of tolerance for extemporaneously compounded pharmaceuticals rests primarily in the samples utilized. These samples were purchased from many practicing pharmacists who are possessed of many degrees of natural skill, and who work under the many different conditions existing in all types of licensed pharmacies. The indicated tolerances are obtained by statistical studies of different series of preparations utilizing the system of least squares and 1.73 standard deviations. The acceptable proportion of samples in each series as determined by this procedure is compared with the acceptable proportion of samples as determined by the points of flexure on probability curves plotted for each series. This comparison indicates that, at least in this type of work, the adopted method yields functionally true tolerances.

THE PROCEDURES employed in these studies and the method of sample collection were described in an earlier paper by the author (1). The data obtained with samples of liquid preparations that were prepared extemporaneously by many licensed pharmacists at their prescription counters are considered in this paper. Samples with deviations that are approximate multiples of the requested weight, or are divisions approximately one-third or more of such weight, have been excluded from consideration in the determination of limits of acceptance or tolerances. Practically all of these extreme deviations can be attributed to mathematical miscalculations, and they should not exert any influence in the determination of standards of tolerance for manipulative precision. The Standard Deviation (S. D.) is based upon devia-

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‡ The conclusions presented in this paper represent the personal opinions of the author.
§ The author is indebted to Mr. J. R. McComas for his assistance.

tions from the theoretical mean and is determined for each series of samples. The S. D. is converted to the equivalent Coefficient of Variation, which is multiplied by the ratio "1.73" to obtain the calculated tolerances for the different preparations. The data for each series are plotted as a "cumulative curve of deviation" and the points of flexure are found by bisection of the angles of tangential intersection. These points are used as comparative provisional limits of acceptance. The calculated tolerance is used in assigning the standard tolerance from a scale which increases by increments of 2.5.

EXPERIMENTAL

All the preparations were tested by the author, using official assays whenever this was possible. Some of the Chloroform Spirit samples were assayed by the procedure which later was adopted by the National Formulary.

The first series contains 191 "selected" samples of Potassium Iodide Solution N. F. (100% *w/v*), which were purchased in $^1/_2$-ounce or 30-cc. quantities. The data are summarized in Table I. The calculated tolerance is ±8.65%, and allows acceptance of 90% of the 195 samples purchased. The points of flexure on the cumulative curve of deviation for this series (Fig. 1) allow acceptance of 89% of all the samples.

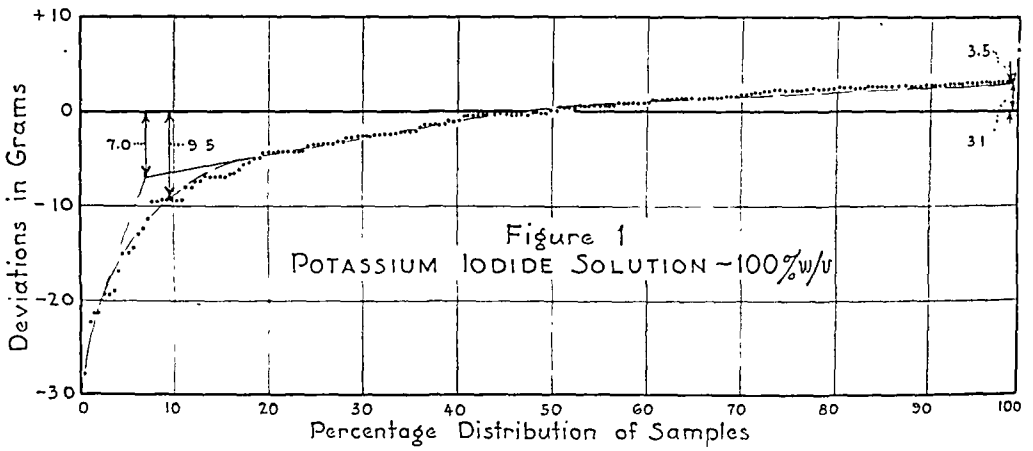


TABLE I.—SUMMARY OF ACCUMULATED DATA

Solution	No. of Samples Purchased	Lowest Concn., %	Concn. of Lower Quartile, %	Concn. of Median, %	Concn. of Upper Quartile, %	Highest Concn., %
Pot. Iodide 100%	195	72.00	96.60	99.60	102.30	106.60
Ammon. Chlor. 19.7%	77	16.22	18.86	19.31	19.70	20.81
Acetic Ac. 10%	64	4.00	9.70	10.20	10.46	13.71
Hydrochlor. Ac. 10%	388	3.64	9.46	9.96	10.36	15.11
Chlorof. Spt. 8.8%	55	4.33	8.28	8.54	8.72	10.65
Pot. Permang. 3%	102	1.00	2.90	3.00	3.10	3.53
Boric Ac. 3%	99	0.95	2.89	2.99	3.15	4.31
Pot. Permang. 2.5%	96	1.38	2.40	2.50	2.60	3.22
Phenol in Oil 10%	44	7.92	9.09	9.80	10.30	19.51
Pot. Permang. 0.5%	64	0.03	0.49	0.50	0.54	0.73
Silver Nitrate 1%	192	0.58	0.97	1.02	1.08	1.59
Phenol in Oil 2%	93	0.62	1.49	1.98	2.12	3.53

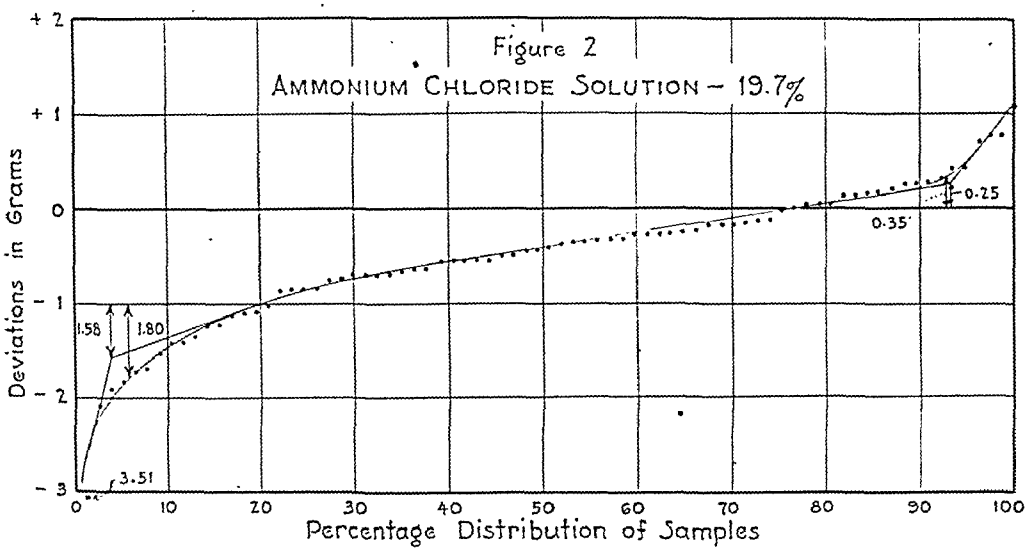
	No. of Samples Purchased	Greatest Minus Dev., %	Dev. of Lower Quartile, %	Dev of Median, %	Dev. of Upper Quartile, %	Greatest Plus Dev., %
Pot. Iodide 100%	195	−28.0	− 3.4	−0.4	+2.3	+ 6.6
Ammon. Chlor. 19.7%	77	−17.8	− 4.4	−2.1	0.0	+ 5.5
Acetic Ac. 10%	64	−60.0	− 3.0	+2.0	+4.6	+37.1
Hydrochlor. Ac. 10%	388	−63.6	− 5.4	−0.4	+3.6	+51.1
Chlorof. Spt. 8.8%	55	−50.8	− 5.9	−3.0	−0.9	+21.0
Pot. Permang. 3%	102	−66.7	− 3.3	0.0	+3.3	+17.7
Boric. Ac. 3%	99	−68.3	− 3.7	−0.3	+5.0	+43.7
Pot. Permang. 2.5%	96	−44.8	− 4.0	0.0	+4.0	+28.8
Phenol in Oil 10%	44	−20.8	− 9.1	−2.0	+3.0	+95.1
Pot. Permang. 0.5%	64	−94.0	− 2.0	0.0	+8.0	+46.0
Silver Nitrate 1%	192	−42.0	− 3.0	+2.0	+8.0	+59.0
Phenol in Oil 2%	93	−69.0	−25.5	−2.0	+6.0	+76.5

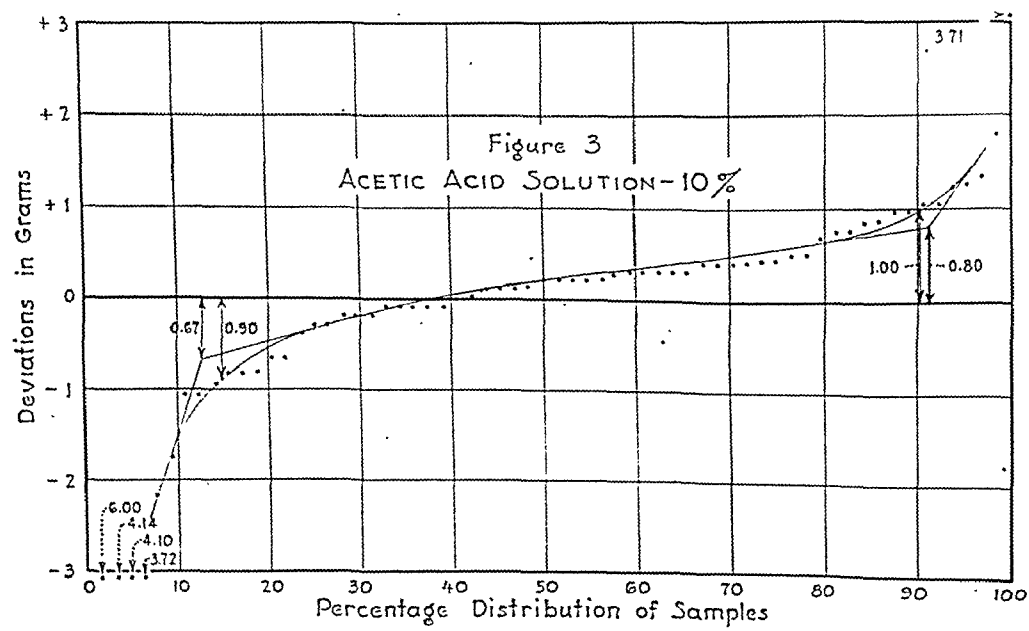
The second series contains 77 samples of Ammonium Chloride Solution, which were requested as: Ammon. Chlor. dr. $4^1/_2$, Water *q. s.* 3 oz. This represents the simplest type of liquid preparation. No calculation is required and a large quantity of the ingredient must be weighed. This is reflected in the fact that no sample in this series was rejected on the basis of excessive deviation. The data are summarized in Table I. The calculated tolerance is ±7.82%, and allows acceptance of 93% of the samples. The points of flexure on the curve for this series (Fig. 2) allow acceptance of 87% of all the samples.

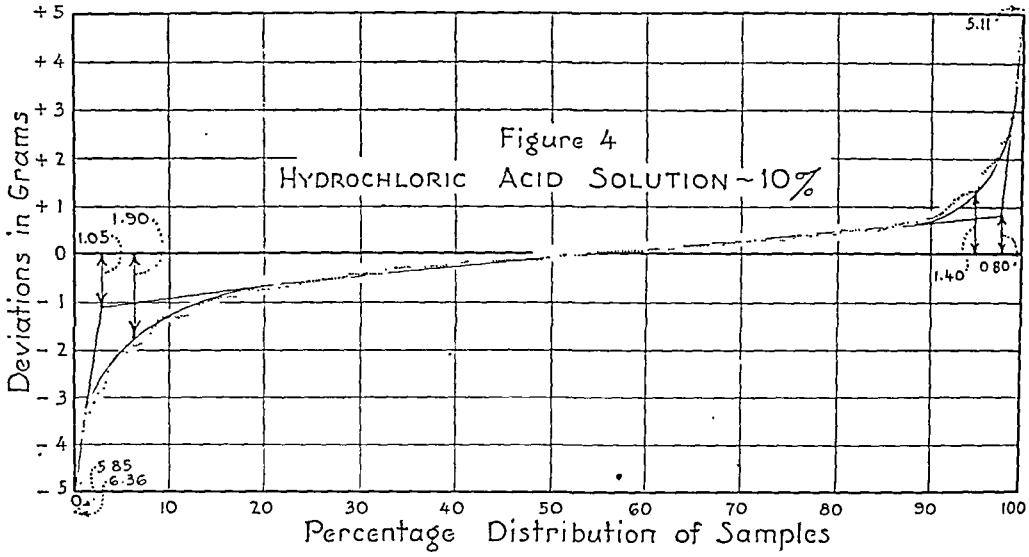
The third series contains 59 "selected" samples of Acetic Acid Solution (10% *w/v*), which were purchased in 2-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±14.54%, and allows acceptance of 88% of the 64 samples purchased. The points of flexure on the curve for this series (Fig. 3) allow acceptance of 77% of all the samples.

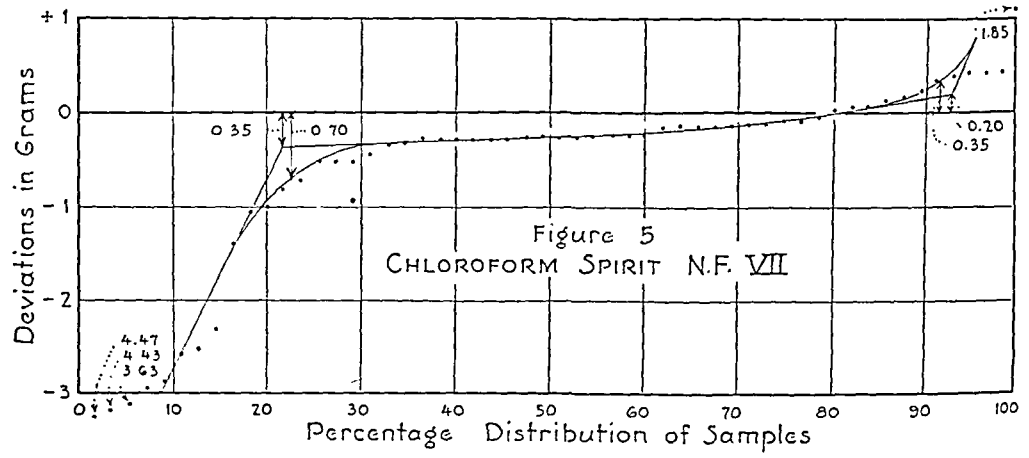
The fourth series contains 377 "selected" samples of Hydrochloric Acid Solution (10% *w/v*), which were purchased in 2-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±15.74%, and allows acceptance of 87% of the 388 samples purchased. The points of flexure on the curve for this series (Fig. 4) allow acceptance of 88% of all the samples.

The fifth series contains 50 "selected" samples of Chloroform Spirit N. F. VII, which were purchased in 2-ounce quantities. The data are summarized









in Table I. The calculated tolerance is ±15.34%, and allows acceptance of 82% of the 55 samples purchased. The points of flexure on the curve for this series (Fig. 5) allow acceptance of 67% of all the samples.

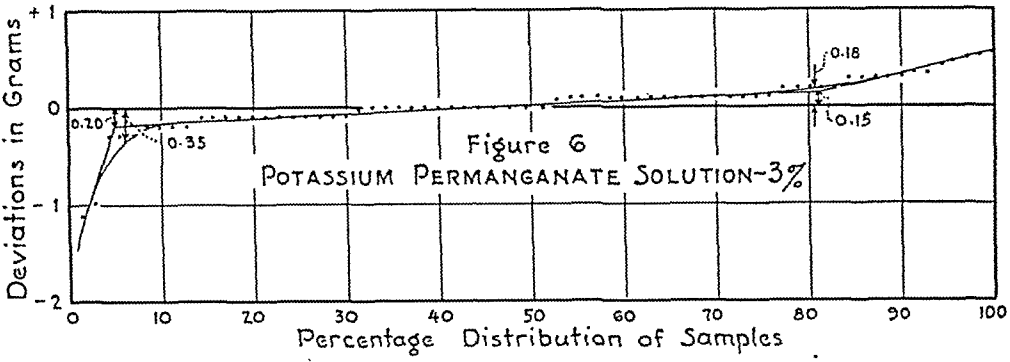
The sixth series contains 95 "selected" samples of Potassium Permanganate Solution (3% *w/v*), which were purchased in 3-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±11.02%, and allows acceptance of 86% of the 102 samples purchased. The points of flexure on the curve for this series (Fig. 6) allow acceptance of 73% of all the samples.

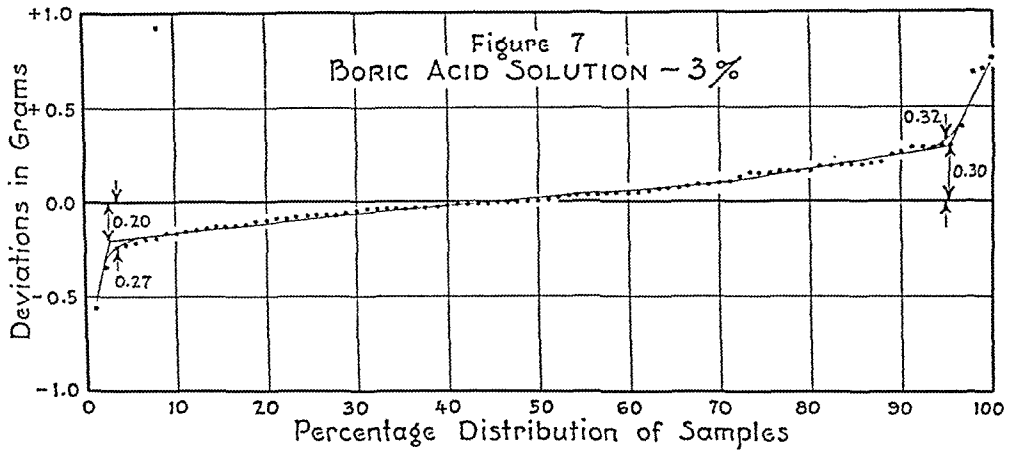
The seventh series contains 90 "selected" samples of Boric Acid Solution (3% *w/v*), which were purchased in 3-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±11.70%, and allows acceptance of 86% of the 99 samples purchased. The points of flexure on the curve for this series (Fig. 7) allow acceptance of 83% of all the samples.

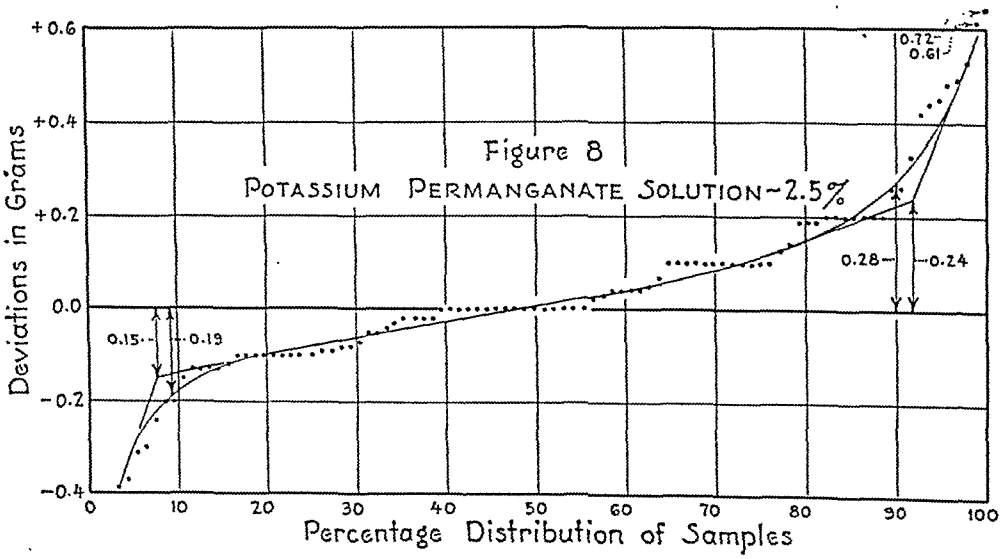
The eighth series contains 94 "selected" samples of Potassium Permanganate Solution (2.5% *w/v*), which were purchased in 3-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±14.0%, and allows acceptance of 88% of the 96 samples purchased. The points of flexure on the curve for this series (Fig. 8) allow acceptance of 81% of all the samples.

The ninth series contains 42 "selected" samples of Phenol in Oil Solution (10% *w/v*), which were purchased in 1-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±15.76%, and allows acceptance of 87% of the 44 samples purchased. The points of flexure on the curve for this series (Fig. 9) allow acceptance of 84% of all the samples.

The tenth series contains 62 "selected" samples of Potassium Permanganate Solution (0.5% *w/v*), which were purchased in 3-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±16.63%, and allows acceptance of



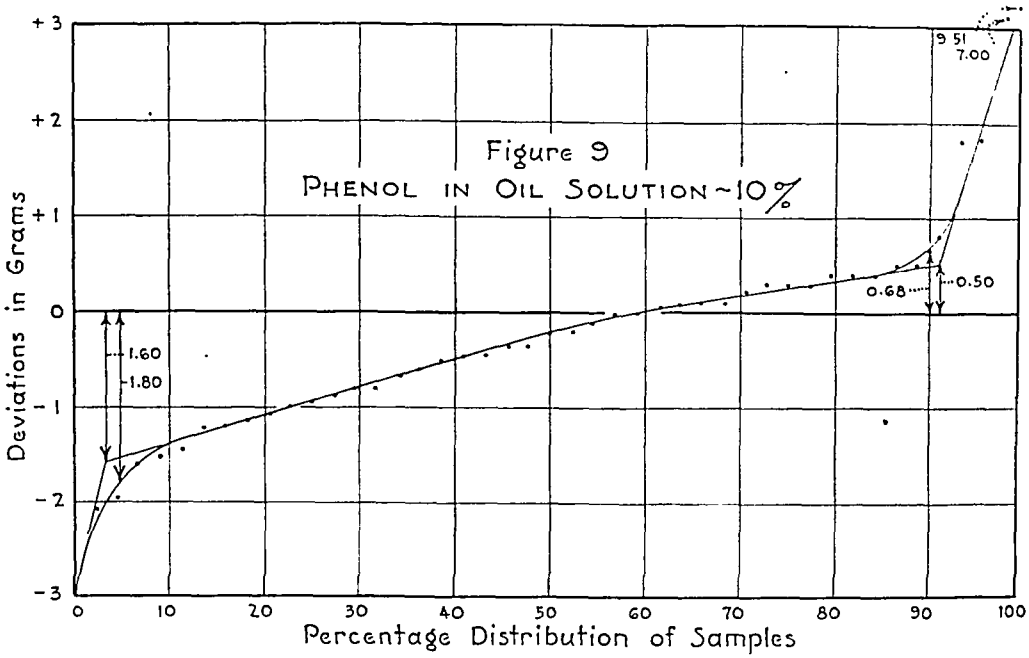


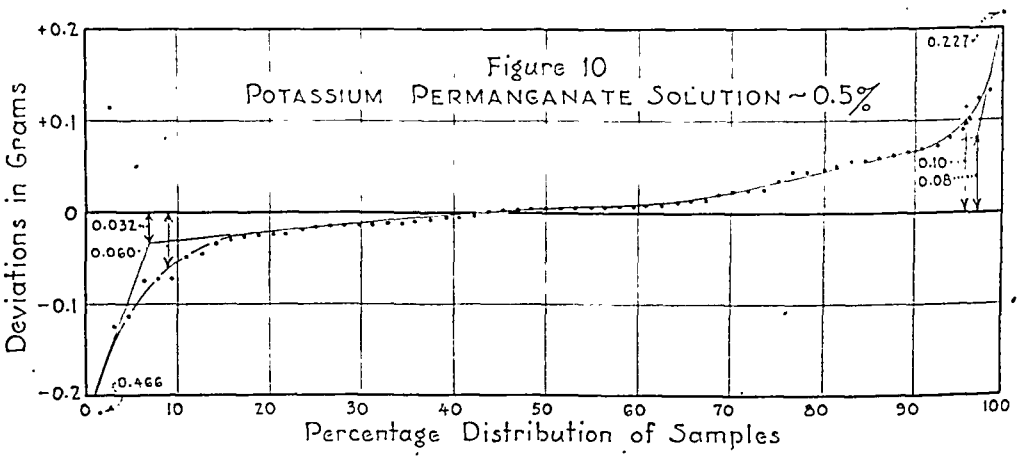


89% of the 64 samples purchased. The points of flexure on the curve for this series allow acceptance of 86% of all the samples.

The eleventh series contains 189 "selected" samples of Silver Nitrate Solution (1% *w/v*), which were purchased in 2-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±15.65%, and allows acceptance of 91% of the 192 samples purchased. The points of flexure on the curve for this series allow acceptance of 92% of all the samples.

The twelfth series contains 69 "selected" samples of Phenol in Oil (2% *w/v*), which were purchased in 1-ounce quantities. The data are summarized in Table I. The calculated tolerance is ±19.26%, and allows acceptance of 66% of the 93 samples pur-





chased. The points of flexure on the curve for this series allow acceptance of 80% of all the samples.

DISCUSSION AND CONCLUSIONS

Under the conditions of these studies, 1.73 standard deviations yield tolerances which appear to represent reasonable and equitable limits of deviation in the compounding of extemporaneous preparations at the prescription counter. These values can be used in establishing standards of tolerance.

It is an undisputed fact that even under controlled conditions, the probability for error increases as the amount of material used decreases. This probability is increased further under prescription-counter conditions. For this reason the solutions are arranged according to the decreasing order of the weights of the medicinal ingredients in Table II.

It is apparent that another factor has disturbed the corresponding increasing order of error as indicated by the determined tolerances. It is also apparent that the solutions which show deviations higher than are indicated by their relative positions, possess the common factor of having an unstable and/or volatile ingredient, such as a volatile acid, chloroform, or phenol. Using the criteria of weight and nature of ingredients, the following classification of liquid preparations is offered for the purpose of establishing standard tolerances.

Group 1. Total weight of ingredient 5 Gm. or more.
(*a*) Stable ingredients.
(*b*) Unstable ingredients.
Group 2. Total weight of ingredient not less than 2.25 Gm. nor more than 4.99 Gm.
(*a*) Stable ingredients.
(*b*) Unstable ingredients.
Group 3. Total weight of ingredient not less than 0.5 Gm. nor more than 2.24 Gm.
(*a*) Stable ingredients.
(*b*) Unstable ingredients.
Group 4. Total weight of ingredient 0.49 Gm. or less.
(*a*) Stable ingredients.
(*b*) Unstable ingredients.

When more than one ingredient is present in a preparation, the total weight and nature of each ingredient shall determine its individual classification.

It is obvious that studies of different preparations placed in the same group and division would yield identical calculated tolerances only by coincidence. A standard tolerance is therefore assigned to each preparation from a scale increasing by increments of ±2.5%. The tolerances assigned to the different groups are derived from the standard tolerances assigned to preparations which unmistakably belong in each particular division. The group assignments and the determined tolerances of the studied solutions are shown in Table II. Using the scale-assigned tolerances for the preparations, the following tentative group tolerances are obtained.

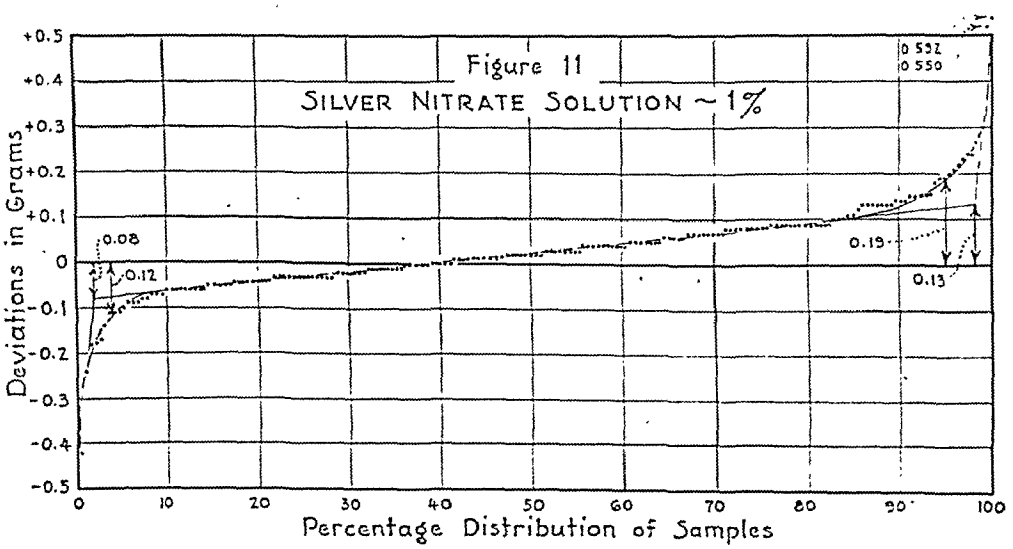
Group 1. (*a*) ±10.0%; (*b*) ±15.0%.
Group 2. (*a*) ±12.5%; (*b*) ±17.5%.
Group 3. (*a*) ±15.0%; (*b*) ±20.0%.
Group 4. (*a*) ±17.5%.

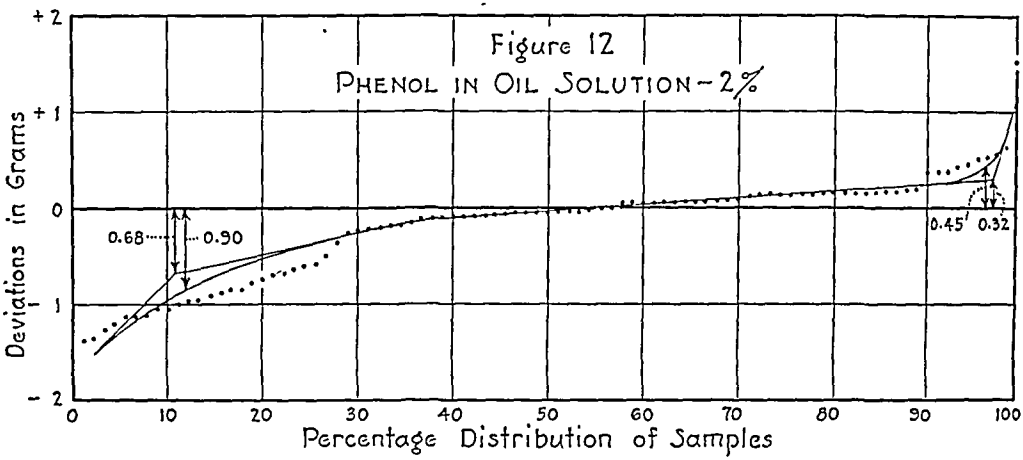
These Standard Tolerances can be applied to any unstandardized preparation containing ingredients that can be accurately determined and are assignable to one of the group divisions.

There is no doubt that further study on a collaborative basis is desirable before standard tolerances for extemporaneously compounded liquid preparations can be officially promulgated. The method presented here appears to yield reasonable and equitable

TABLE II.—CLASSIFICATION AND TOLERANCES OF LIQUID PREPARATIONS

No. of Selected Samples	Solution	Wt. of Ingredient Requested, Gm.	Group Placement	Calcd. Tolerance, ±%	Assigned Tolerance, ±%
77	Ammon. Chlor. 19.7%	17.50	1 (*a*)	7.82	7.5
191	Pot. Iodide N. F.	15.00	1 (*a*)	8.65	10.0
59	Acetic Ac. 10%	5.91	1 (*b*)	14.54	15.0
377	Hydrochlor. Ac. 10%	5.91	1 (*b*)	15.74	15.0
50	Chlorof. Spt. N. F.	5.32	1 (*b*)	15.34	15.0
42	Phenol in Oil 10%	2.96	2 (*b*)	15.76	17.5
95	Pot. Permang. 3%	2.66	2 (*a*)	11.02	12.5
90	Boric Ac. 3%	2.66	2 (*a*)	11.70	12.5
94	Pot. Permang. 2.5%	2.22	3 (*a*)	14.00	15.0
190	Silver Nitrate 1%	0.59	3 (*a*)	15.65	15.0
69	Phenol in Oil 2%	0.59	3 (*b*)	19.26	20.0
62	Pot Permang. 0.5%	0.44	4 (*a*)	16.63	17.5





tolerances, and it is recommended that it be used as a basis for the establishment of such standards. These tentative standards are also recommended to the Food and Drug Officials of the individual states as an aid in judging the quality of prescription-counter products on a reasonable and equitable basis.

SUMMARY

1. Tolerances are determined for twelve liquid preparations by means of the Standard Deviation based on the least squares. The percentages of the samples acceptable by this method are compared with the values determined by the points of flexure on serial curves of deviation.

2. The Coefficient of Variation equivalent to 1.73 Standard Deviations of an adequate series of samples is recommended as a reasonable and equitable tolerance.

3. A system of classification of liquid preparations based upon the weight and nature of the ingredients is described.

4. A system for assigning Standard Tolerances from the determined tolerances, and the application of these Standard Tolerances to any preparation meeting the classification requirements are described.

5. Tentative Standard Tolerances have been determined for the different classifications.

6. It is recommended that the tentative Standard Tolerances should be used by Food and Drug Officials as a reasonable and equitable basis for their decisions.

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The Assay of Relatively Pure Benzylpenicillin by Ultraviolet Absorption*

By A. A. COLON, G. E. HERPICH, J. D. NEUSS, and H. A. FREDIANI

The results of a comparative study of three spectrophotometric methods currently in use for the assay of benzylpenicillin are reported.

THE CURRENT official F. D. A. method (1) is still the sole, truly specific chemical method for the quantitative estimation of benzylpenicillin (penicillin G). This method, reported in detail by Mader and Buck (2) and Sheehan, Mader, and Cram (3), is least subject to interference by other penicillins and by extraneous materials. However, since this method requires two to four hours per analysis, a more rapid screening method for relatively high purity material is desirable for routine controls in the production of salts of benzylpenicillin. The fortuitous presence of a phenyl group in benzyl-

* Received Sept. 29, 1948, from the Chemical Control Division, Analytical Research Department, Merck & Co. Inc., Rahway, N. J.

penicillin has led to a variety of ultraviolet absorption methods for the quantitative estimation of this important antibiotic.

Herriott (4) was the first to publish an ultraviolet method for penicillin estimation. His method utilized the relatively high absorption at 322 mμ of a degradation product of penicillin as a rapid method for penicillin estimation. In this method, the absorption of the original solution is measured at 322 mμ; degradation of the penicillins present is effected by heating for 15 minutes at 100° in 0.3 *M* acetate buffer (*p*H 4.6) and the increase in absorption at this same wave length is measured. Although rapid, this technique does not differentiate between the various possible penicillins and is apparently useful only in determining the total quantity of penicillins present in solution over the range of 5 to 75 μg. with a reported accuracy of ±5%.

Philpotts, Thain and Twigg (5) suggested the use of the ultraviolet absorption of the phenyl group for the estimation of benzylpenicillin in the presence of other penicillins. The choice of a spectrographic technique, employing a wedge cell and photographic plate matching, is inconvenient for the analytical laboratory since the procedure is more cumbersome and time-consuming than the utilization of modern spectrophotometers designed for absorption studies.

Grenfell, Means and Brown (6) have recently described a convenient and rapid ultraviolet absorption method developed in the Charles Pfizer & Co., Inc., Laboratories, for the determination of benzylpenicillin. Their procedure involves:

(*a*) Use of specific rotation of the relatively pure penicillin solution to be tested as a measure of the *total* penicillins present.

(*b*) Measurement of the characteristic ultraviolet maxima of the benzyl group at 258 and 263 mμ to determine the benzylpenicillin present.

These authors recognize the possible interference of parahydroxy benzylpenicillin (penicillin X) and use Macpherson's (7) modification of the Pauly reaction to note the presence or absence of this penicillin in the sample studied. Their method involves the measurement of the optical density of the solution at 263 mμ and subtraction from this value of the optical density at 280 mμ. The latter is taken as a measure of degradation products and interfering substances which would increase the 263 absorption over and above that for the benzyl group present. This correction is found to be applicable, at the concentrations used, only when the density at 280 mμ is 0.10 or less; at higher values it is recommended that the sample be purified by conversion to the ammonium salt.

In his discussion on "A System of Penicillin Analysis" at the Chicago meeting of the American Chemical Society on April 21, 1948, G. B. Levy (8) reviewed in some detail a spectrophotometric technique developed in the Schenley Laboratories for estimation of benzylpenicillin. This method utilized the benzyl group absorption bands (in 95% ethanol solution) at 264.5 and 268.5 mμ. The difference in absorption between the peak at 264.5 mμ and the dip at 263 mμ is taken as a measure of the benzylpenicillin present. By measuring this difference the interference caused by extraneous absorbing material is minimized. Since a comparatively large temperature effect was found, constant temperature should be maintained in the absorption cell compartment.

In the Merck laboratories an ultraviolet method (9) employing more specific but limited corrections for possible common degradation products has been in routine use for about two years. The correction is based upon the thorough study of the ultraviolet absorption of the penicillins and their degradation products made by N. R. Trenner (10). These studies have indicated that the most common degradation products of penicillins are penicillenic acid and penamaldates. The E_m for penicillenic acid is 28,000 at its maximum at 320 mμ, and 885 at 257 mμ. The E_m for penamaldates is about 17,000 at its maximum at 280 mμ and 7500 at 257 mμ. Thus, it is possible by three precise absorption readings: (1) to measure at 257 mμ the sum of the absorption due to benzylpenicillin (at its maximum) together with the residual absorption due to the *usual* major degradation products, (2) to measure the absorption due to penicillenic acid at its maximum and thus calculate its effect on the benzyl peak at 257 mμ, and (3) to measure the absorption due to penamaldates at their absorption maximum and thus calculate their effect on the benzyl peak. Since the ratio of the absorptions for penicillenic acid at 257 mμ and at 320 mμ is 885:28,000, a factor of 1:30 is sufficiently precise for correction purposes. For penamaldates the ratio of 7500 to 17,000 approximates a 1:3 factor. In this method, then, the absorption of the solution of the relatively pure sample is measured at 257, 280, and 320 mμ. One-third of the measured density for the solution at 280 mμ and one-thirtieth of the measured density at 320 mμ are deducted from the measured density at 257 mμ. This results in a value closely approximating the absorption contributed by the benzyl group at 257 mμ. It should be borne in mind that penicilloates (10) present will be measured, of course, as penicillin.

In the above methods it is important to remember that the use of any ultraviolet absorption method is valid only in the absence of significant amounts of ultraviolet absorbing impurities. The benzylpenicillin content is measured indirectly through the benzyl absorption. Any aromatic impurity will have a large effect upon the precision of a benzylpenicillin assay by ultraviolet absorption technique. Such impurities are possible since substances like phenylacetic acid are frequently added to fermentation broths in order to increase the production of the benzyl form (11).

As far as other penicillins are concerned, the presence of parahydroxybenzylpenicillin (containing a phenol group) will definitely interfere with the determination of benzylpenicillin. Although other commonly occurring penicillins do not show the characteristic benzyl absorption peaks it should be pointed out that their ultraviolet absorption is appreciable at the lower wave lengths used (Fig. 1). Thus their presence, unless corrected by readings at other wave lengths, would tend to yield erroneously high values for benzylpenicillin. It is always advisable, in an assay of this type, to verify the actual presence of the benzyl peak by taking sufficient readings. Also, due to their microbiological activity, these other penicillins will disrupt correlation between ultraviolet and microbial assays.

In spite of these limitations, however, the ultraviolet absorption method for benzylpenicillin assay has been extremely useful in the production of high purity material because of its rapidity and reproducibility. Therefore, a comparative study of the various modifications suggested has been made in this laboratory. This study should be of interest to other laboratories because of the clinical importance of this antibiotic.

EXPERIMENTAL

A carefully tested standard sample of crystalline benzylpenicillin sodium was used to ascertain adherence to Beer's law. This sample had been in continual use as a standard in this laboratory and consequently many determinations had been performed on it by microbial means and by the official F. D. A. precipitation method. The average purity found for this sample by the F. D. A. N-ethyl piperidine method was 96.5 ± 0.3%. Low absorption at wave lengths above 270 mμ indicates a paucity of degradation products and a sensitive phenol test indicates the presence of less than 0.1% parahydroxybenzylpenicillin. An absorption curve of this sample is shown in Fig. 1.

1. Pfizer Method (Modified).—Six standard solutions of the above sample were prepared in the range 15 to 100 mg. benzylpenicillin per 100 ml. of solution, using distilled water at 25° as solvent The absorption was immediately measured at 263 and 280 mμ The arithmetical difference between these two values was then plotted against the concentration taken. The results are shown in Table I and Fig. 2.

The excellent straight line indicates rigid adherence to Beer's law under these conditions. The "unknowns" to be tested were then weighed out, dissolved in water, readings taken at 263 and 280 mμ, and the apparent benzylpenicillin content determined from this graph. The purities were calculated by dividing the benzylpencillin contents, as read from the graph, by the actual weights of samples taken. It should be noted that this constitutes a modification of the published method (6), which estimates the total penicillin by optical rotation. The use of

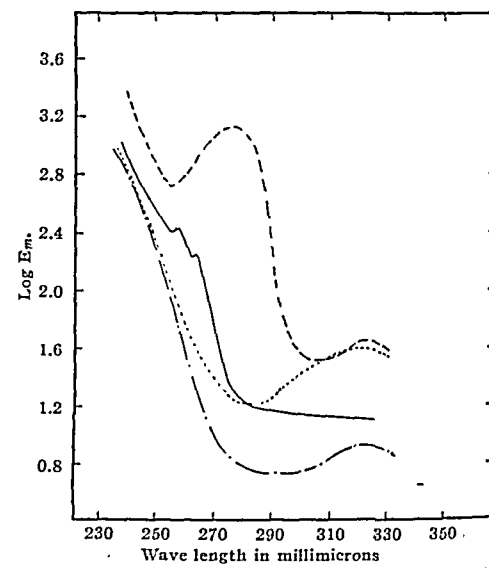

Fig. 1.—Ultraviolet absorption curves of the penicillins. Sodium Salt of: —— Penicillin G; ---- Penicillin X; ----- Penicillin K; —·— Penicillin F.

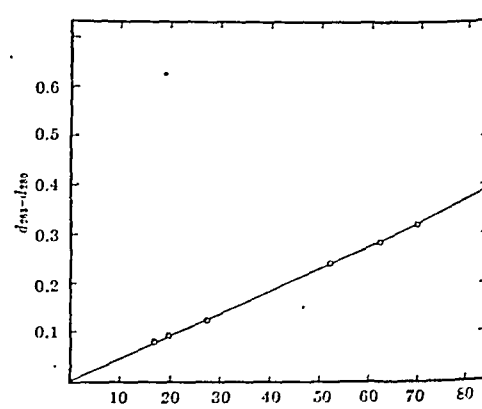

Fig. 2.—Calibration curve for the modified Pfizer method.

TABLE I.—DATA FOR STANDARD CURVES

Mg./100 Ml.	d_{257}	d_{263}	$d_{264.5}$	d_{280}	d_{320}
Aqueous					
16.8	0.139	0.091	...	0.010	0.006
19.9	0.148	0.105	...	0.012	0.009
27.5	0.209	0.136	...	0.010	0.010
51.7	0.395	0.262	...	0.018	0.013
61.8	0.472	0.309	...	0.023	0.020
69.5	0.520	0.339	...	0.019	0.018
Alcoholic					
15.0	...	0.064	0.069	...	...
20.1	...	0.085	0.094	...	...
25.0	...	0.105	0.117	...	...
31.2	...	0.131	0.142	...	...
33.5	...	0.141	0.159	...	...
34.9	...	0.140	0.159	...	...
43.0	...	0.181	0.199	...	...
44.8	...	0.187	0.212	...	...
48.7	...	0.202	0.228	...	...
51.1	...	0.211	0.237	...	...
67.7	...	0.287	0.312	...	...
89.7	...	0.377	0.421	...	...
139.9	...	0.589	0.652	...	...
190.0	...	0.786	0.876	...	...
230.4	...	0.958	1.061	...	...

a weighed sample avoids the inherent errors in carrying out rotation measurements and takes into account optically inert impurities.

2. Schenley Method (Modified).—Since alcoholic solutions are recommended in this method, additional portions of the standard sample were weighed out covering the range of 15 to 250 mg. per 100 ml., using 95% ethanol as solvent. The differences between the absorptions at 264.5 and 263 mμ were plotted against concentration taken. This is shown in Table I and Fig. 3. Here again adherence to Beer's law is indicated. The "unknowns" were then analyzed by dissolving weighed samples, measuring their absorption at the above wave lengths, and ascertaining, from the graph, the apparent benzylpenicillin content of the solution.

Since our laboratory deals primarily with highly purified samples, a simplification of the Schenley Method (8) is in order. Depending on the type of impurities present, the original method frequently indicates a negative intercept on the ΔE axis of the calibration curve. Our simplification assumes, in essence, that this intercept is zero. In samples assaying above 95% the error due to this simplification is less than 1%.

3. Merck Method.—In addition to the absorption values (263 and 280 mμ) measured on the samples for the "Pfizer Method," the absorption of these solutions was also measured at 257 and 320 mμ, as shown in Table I. One-third of the measured value at 280 mμ and one-thirtieth of the measured value at 320 mμ were deducted from the value at 257 mμ and the resultant value plotted against concentration. Figure 4 again indicates adherence to Beer's law. The "unknowns" were tested by reading the apparent benzylpenicillin content from the graph and comparing this value with the actual sample weight.

The precision of the three methods was ascertained by replicate determinations on a sample of benzylpenicillin which assayed 91.0% by the F. D. A. precipitation method. The results are summarized in Table II.

TABLE II.—PRECISION OF METHODS USED

Mg./100 ml.	Benzylpenicillin Content, %	Error, %
Schenley Method		
21.3	84.5	−6.5
55.2	97.4	+6.4
101.3	89.0	−2.0
102.5	91.5	+0.5
107.7	97.8	+6.8
116.6	88.5	−2.5
114.7	94.0	+3.0
130.2	89.0	−2.0
130.4	90.5	−0.5
149.5	94.5	+2.5
Pfizer Method		
18.8	93.0	+2.0
24.9	94.5	+3.5
36.3	93.5	+2.5
41.4	92.5	+1.5
47.7	92.5	+1.5
66.2	93.0	+2.0
69.4	91.5	+0.5
83.6	92.0	+1.0
Merck Method		
18.8	97.5	+6.5
24.9	98.0	+7.0
36.3	94.5	+3.5
41.4	94.5	+3.5
47.7	93.5	+2.5
66.2	94.5	+3.5
69.4	93.5	+2.5
83.6	93.5	+2.5

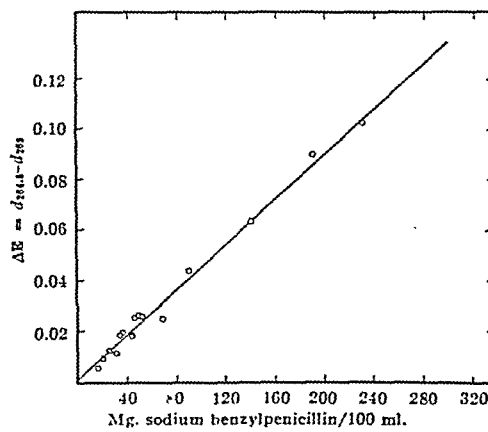

Fig. 3.—Calibration curve for the modified Schenley method.

The comparative accuracy of the three methods was determined by testing a group of samples that had been previously assayed by the official F. D. A. method. The results are given in Table III.

DISCUSSION

Statistical analysis of the above data (by J. H. Davidson of this Company) resulted in the following conclusions:

(a) A regression line for each of the calibration curves of the three methods gave y intercepts which were well within the error of determination of show-

TABLE III.—ACCURACY OF METHODS

Sample No.	F. D. A. Method, % G	Pfizer Method % G	Pfizer Method % Error	Schenley Method % G	Schenley Method % Error	Merck Method % G	Merck Method % Error
1	94.5	96.5	+2.0	90.0	−4.5	98.0	+3.5
2	94.5	95.0	+0.5	85.5	−9.0	96.5	+2.0
3	95.5	94.5	−1.0	..	...	97.5	+2.0
4	96.5	97.5	+1.0	89.0	−7.5	96.5	+0.0
5	96.0	97.5	+1.5	92.5	−3.5	98.0	+2.0
6	90.0	92.0	+2.0	89.0	−1.0	93.5	+3.5
7	94.5	95.0	+0.5	93.0	−1.5	94.0	−0.5
Av.	94.5	95.4	1.2	89.8	4.5	96.3	1.9

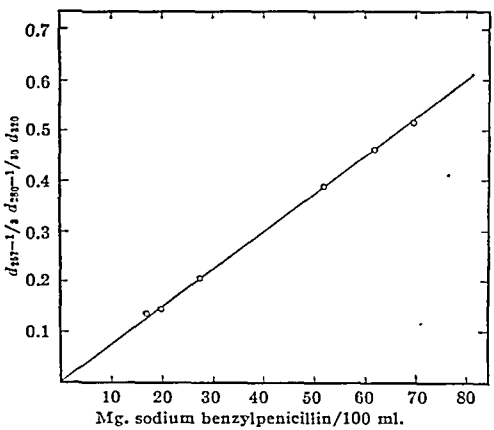

Fig. 4.—Calibration curve for the Merck method.

ing a zero density reading for zero concentration. This is in accordance with Beer's Law.

(*b*) The slopes of these calibration curves, which may be defined as the change in density function of the solution for each milligram increase in benzylpenicillin concentration (per 100 ml.) were found to be:

Pfizer	0.00460 ± 0.00006
Schenley	0.00045 ± 0.00003
Merck	0.00746 ± 0.00003

The ± figure is the range within which the slope is known with a probability of 0.99. Of the three methods the Merck procedure gives the largest density change per unit concentration, and the smallest relative error in precision.

(*c*) Using the data from Table III, the average difference between each method and the official F. D. A. chemical method, together with the expected range (probability of 0.99) within which the difference should fall, is given in Table IV.

CONCLUSIONS

Of the four methods suggested in the literature for ultraviolet assay of benzylpenicillin salts, the photographic has not been included for manipulatory reasons. Of the three spectrophotometric methods the one described by Grenfell, *et al.* (6) gives the best agreement with the official F. D. A. precipitation method. For routine controls the Merck method is also satisfactory. Two factors militate against the Schenley method. The necessary careful temperature control does not add to the attractiveness of this method; also, this method utilizes small differences between two relatively high optical density measurements. As may be seen from Fig. 3 an optical density difference of 0.005 unit represents 5% in the final assay. Although the Beckman spectrophotometer is a good instrument, its reproducibility is no better than 0.005 unit at the densities measured.

TABLE IV

Method	Average Difference from F. D. A., %	Difference Range
Pfizer	0.9	−1.8 to +3.7
Schenley	−4.5	−12.8 to +3.8
Merck	1.8	− 2.2 to +5.8

SUMMARY

A comparative study has been made of three spectrophotometric methods currently in use for assay of benzylpenicillin.

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A Chromatographic Study of Pyrethrum Flowers*,†

By G. H. HAMOR, J. F. SUCHY, and C. H. WALDON

This work consists of an investigation of the adsorption of extracts of Chrysanthemum (*Pyrethrum*) *Cinerariaefolium* on a chromatographic column. The study was undertaken to determine the possibility of the separation of a pyrethrum extract mixture on a chromatographic column into different colored bands, with the subsequent identification, estimation, and isolation of the component parts (especially pyrethrins and cinerins) of the extract.

SINCE THE introduction of pyrethrum as an insecticide in the middle of the nineteenth century, an intensive investigation has been undertaken to determine its active principles and to find an accurate method of quantitative analysis. Recent work (1) seems to indicate that the toxicity is due to four compounds instead of the two pyrethrins previously considered the only active principles. These four constituents are the two esters of chrysanthemum monocarboxylic acid with the cyclic ketonic alcohols, pyrethrolone and cinerolone; and the two methyl esters of chrysanthemum dicarboxylic acid with these alcohols.

Because the present chemical methods of determining pyrethrin content are relatively long and complicated with many chances for error, this study of adsorption analysis of pyrethrum extracts was undertaken to determine the possibility of resolving and quantitatively separating the pyrethrins by means of chromatography.

EXPERIMENTAL

The material used consisted of flowers of *Chrysanthemum cinerariaefolium* grown in the drug garden of the School of Pharmacy, Montana State University. Using the National Formulary method of analysis (2) the flowers averaged 0.81% Pyrethrin I and 0.58% of Pyrethrin II.

Alumina was first tried as an adsorbent. Several organic solvents were used to extract the pyrethrum flowers. A petroleum ether extract gave an orange-red band which could not be developed or eluted.

Powdered sucrose, Lloyd's Reagent, Celite, calcium oxide, calcium hydroxide, calcium carbonate, bentonite, Permutit and magnesium carbonate were tested as adsorbing agents and were found to be unsatisfactory. Heavy magnesium oxide of the ordinary commercial grade gave the most well-defined bands. The extract used was prepared by subjecting a 492.5-Gm. sample of *Chrysanthemum cinerariaefolium* to twenty hours of continuous extraction in a Friedericks Extractor with petroleum ether as a solvent and evaporating the resulting solution to exactly 200 cc. This extract assayed 0.007360 Gm. of Pyrethrin I per cc. and 0.006614 Gm. of Pyrethrin II per cc. Thirty cubic centimeters of this solution was adsorbed on a magnesium oxide column 25 mm. in diameter. Three zones were observed: a top band, greenish yellow in color; a middle zone, orange-yellow in color; and a colorless bottom band.

Various solvents were tested in the development of the chromatogram, none being highly successful. In most cases a pink-colored layer was observed but this band could never be distinguished in the extruded column. Different eluents were tried but elution could be obtained only by subjecting the dry powder to twenty hours of continuous extraction with petroleum ether. On analysis of the different zones, approximately 65% of the pyrethrins were found to be in the middle orange-yellow layer, with 20% in the top greenish yellow band, and a negligible amount in the colorless band. Pyrethrins I and II were present in approximately the same percentage. Eighty-five per cent recovery from the column was obtained.

In an effort to attain further concentration, the orange-yellow band containing the bulk of the pyrethrins was extracted and run through another chromatographic column. No distinctive layers were obtained, the entire column assuming a homogeneous bright yellow color.

In order to rule out the possibility of other plant principles interfering with the adsorption of the pyrethrins, it was necessary to obtain the pyrethrins in a purified form. Starting with a soluble pyrethrum oleoresin,[1] and using the method of Barthel, Haller, and La Forge (3), a concentrate containing 90% of total pyrethrins was prepared.

Of this concentrate, 1.5 Gm. was diluted to 25 cc. with petroleum ether. Two cubic centimeters of this solution was run through a column of heavy magnesium oxide. A distinct pink upper layer 2 mm. in width was formed and, on standing or developing, a very light red lower band 50 mm. in width was seen. Differentiation of the layers could not be attained. Elution was obtained only by dissolving the dry powder with 10% hydrochloric acid. The pyrethrins were then extracted by shaking with petroleum ether. On analysis, 29% of the pyrethrins were found in the pink layer and 69% in the lower red layer, thus effecting a total recovery of 98%.

* Received Sept. 2, 1948, from the School of Pharmacy, Montana State University, Missoula, Mont.

† School of Montana State University by Glenn H. Hamor in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

[1] S. B. Penick Co., 50 Church St., New York 7, N. Y.

SUMMARY AND CONCLUSIONS

When a pyrethrum extract was chromatographed, the bulk of the pyrethrins was concentrated in a highly colored orange-yellow zone which could not be differentiated.

When the extract was freed of most of the interfering plant principles and run through a column, two differently colored zones, one dark pink and the other light pink in color, were observed. Both of these zones contained pyrethrins.

An effective resolution of pyrethrins could not be obtained by chromatography, possibly due to the fact that the pyrethrins, being esters, were decomposed by the alkalinity of the magnesium oxide. This hydrolysis would not be detected on analysis because the assay for pyrethrins is based on the reaction with acids freed from the esters.

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Modified Assay Methods for Crude Drugs Involving the Removal of Interfering Substances by Enzymatic Digestion. II. Modified Assay Method for Belladonna Root*

By HENRY LOUIS VERHULST,† LOUIS ARRIGONI,‡ and LOUIS FISCHER§

The N. F. method for the determination of the alkaloid content was modified by digesting the drug with a mixture of "Taka-Diastase" and pancreatin. After digestion, the mixture was extracted with chloroform and the official method was carried out to completion. Satisfactory results were obtained with a material improvement in the procedure from the standpoint of decreasing the time, equipment involved, and the facility of operation.

THE OFFICIAL ASSAY of belladonna root requires maceration of the drug overnight followed by Soxhlet extraction for three hours, or an alternate percolation method. In either process the alkaloids are separated and purified by the immiscible solvent procedure. The alkaloids are then determined volumetrically using 0.02 *N* acid and alkali. The main objections to the official assay are: (*a*) the length of time required; (*b*) the tendency to form emulsions during the shaking-out process; and (*c*) the extraction of relatively large quantities of impurities along with the alkaloids.

This study was undertaken to develop a process which would eliminate the use of Soxhlet equipment, overnight maceration, some of the emulsions, impurities from the extractions, and still yield satisfactory results. The method of approach chosen to remedy these objections and to simplify the official assay was to hydrolyze enzymatically the proteins and carbohydrates, and in this manner attempt to eliminate the extraction of interfering substances and impurities.

EXPERIMENTAL

Before undertaking the investigation of a new process using enzymes,[1] it was first necessary to have information regarding the composition of the drug; consequently, a partial phytochemical analysis of the sample was made.

The drug, No. 40 powder belladonna root, was thoroughly mixed and placed in a tightly closed

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[1] Subsequent to the presentation of this paper for publication the following reference, "Utilisation of Diastase in the Extraction of Alkaloids from Crude Drugs," by H. N. Gupta and S. B. Sen Gupta appeared in *Chemical Abstracts*, 42, 6059b (Aug. 20, 1948). This work involved a similar study on the use of enzymatic degradation of cellular material for the purification of the alkaloids in several plant drugs. *Priority is claimed for this type of investigation by virtue of* the master's thesis by A. J. Anderson, entitled "A Study of Assay Methods for Colchicum Corm and Seed Including the Removal of Interfering Substances by Enzymatic Digestion," which was accepted by the University of Washington Library, June, 1946.

container. All determinations using official methods were made in triplicate (1), or until satisfactory results were obtained. The values, calculated on a moisture-free basis, are reported in Table I.

TABLE I.—PARTIAL PROXIMATE COMPOSITION BELLADONNA ROOT

	%
Moisture	6.43
Starch	38.45
Reducing Sugars	2.35
Sucrose	12.20
Ash	11.70
Protein	6.73
Petroleum Ether Extract	0.47
Pentosans	None

For comparative purposes, the alkaloidal content of the drug was determined by the N. F. VIII procedure (2) and the results are presented in Table II.

TABLE II.—RESULTS OF N. F. ASSAY OF BELLADONNA ROOT

Sample	%
1	0.33
2	0.32
3	0.35
4	0.35
5	0.35
6	0.34
7	0.35
Av.	0.34

Assay of Belladonna Root by Enzymatic Digestion with Pancreatin and Taka-Diastase.—The selection of suitable enzymes to hydrolyze the starch and proteins in belladonna root was limited to pancreatin and "Taka-Diastase." Gelatinizing the starch in the drug by heating in a boiling water bath before subjecting it to the action of pancreatin and subsequent extraction of the active constituents gave low results, probably because of hydrolysis of the alkaloids. In an effort to overcome the decrease in alkaloidal yield, the procedure was modified by eliminating the preliminary heating. The large amount of starch present indicated that Taka-Diastase could be used to advantage as the Taka-Diastase digests about 300 times its weight of starch while pancreatin digests 25 times its weight of the carbohydrate. The use of the pancreatin-Taka-Diastase mixture required a shorter digestion time than pancreatin alone.

In the preliminary treatment of the samples with enzymes, three different methods were used. First, a 10-Gm. sample was mixed with 20 cc. of *p*H 6.7 sodium phosphate buffer and 30 cc. of water. The mixture was then placed in a beaker of boiling water for twenty minutes to gelatinize the starch. After cooling, 1.5 Gm. of pancreatin was added and the mixture digested at 55° for two and a half hours, at which time a drop of iodine T. S. gave no blue color when added to one drop of the homogeneous mixture. The second method was similar to the above except that the sample was not heated. The third procedure was identical with the latter save that 0.5 Gm. of Taka-Diastase was added in addition to the pancreatin and the digestion time was reduced to two hours.

After complete digestion in each instance, the sample was transferred quantitatively to a separatory funnel. Several organic solvents were used to determine which was the best for the extraction of the alkaloids. Chloroform showed the least tendency to emulsify and consequently was adopted for the procedure which follows.

Fifty cubic centimeters of chloroform was added to the separatory funnel; the solution was neutralized with ammonium hydroxide; and an excess of 2 cc. was added. The alkaloid was then extracted with the organic solvent and the chloroform layer drawn off and passed through a dry filter into a second separatory funnel. The alkaloid was extracted with three more 25-cc. portions of chloroform. Two cubic centimeters of the final chloroform extraction was evaporated to dryness, the residue was taken up in several cubic centimeters of 0.1 *N* sulfuric acid and tested with Valser's reagent. The fourth extraction always gave a negative test for alkaloids. The combined chloroform extractions were then evaporated to about 50 cc. and extracted with three 25-cc. portions of 0.5 *N* sulfuric acid. The combined sulfuric acid extractions were neutralized and shaken out with chloroform. The total chloroform extractions were evaporated on a water bath to dryness and the flask heated for fifteen minutes. The residue was redissolved in a small volume of the solvent, evaporated to dryness on a water bath, and again heated for fifteen minutes. This treatment was repeated for a third time. The alkaloids in the residue were determined as directed in the N. F. VIII. The results obtained from the extraction after the pancreatin-Taka-Diastase digestion were comparable with those obtained by the N. F. method as may be observed in Table III.

Effect of Enzymes on Loss of Atropine.—In reviewing the literature, it was noted that loss of atropine by hydrolysis in the presence of natural enzymes was reported (3). To determine if any such loss of alkaloid occurred during the digestions with Taka-Diastase and pancreatin, a known quantity of atropine was added to three 10-Gm. samples of Belladonna root and analyzed.

To insure that the atropine used was pure, 200 mg. was made up to 200 cc. in a volumetric flask with a known quantity of 0.02 *N* sulfuric acid and a 50-cc. aliquot was titrated using standard alkali. This determination in duplicate showed each 50-cc. portion contained 50.1 mg. of atropine. Ten cubic centimeters of the remaining solution was added to each of two samples and 20 cc. to a third sample. The samples were then analyzed for atropine by the procedure described above.

The results, as shown in Table IV, indicate there was no loss of alkaloid during the enzymatic digestion.

SUMMARY

The work was divided into two parts: (*a*) partial phytochemical analysis of the drug and (*b*) the study of enzymatic digestion of the root followed by the extraction of the alkaloids from the digested mixture.

TABLE III.—COMPARISONS OF RESULTS OBTAINED BY N. F. AND PROPOSED METHODS

Sample	N. F. Method, %	Pancreatin Modification with Heat, %	Pancreatin Modification without Heat, %	Pancreatin-Taka-Diastase Modification, %
1	0.33	0.29	0.35	0.36
2	0.32	0.30	0.34	0.35
3	0.35	0.30	0.35	0.35
4	0.35	..	..	0.36
5	0.35	..	..	0.35
6	0.34	..	..	0.35
7	0.35	..	..	..
Av.	0.34	..	..	0.35

TABLE IV.—COMPARISON OF ATROPINE RECOVERED FROM SAMPLE WITH KNOWN QUANTITY ADDED

Weight of Sample, Gm.	Alkaloidal Content of Sample, Mg.	Amount of Atropine Added to Sample, Mg.	Total Alkaloid Extracted, Mg.	Amount of Added Atropine Recovered, Mg.
10.6442	36.2	10	46.4	10.2
10.2657	34.9	10	44.7	9.8
10.7330	36.5	20	56.7	20.2

The official method for the determination of the alkaloid content was modified by digesting the drug with a mixture of Taka-Diastase and pancreatin. After digestion, the mixture was extracted with chloroform and the official method was carried out to completion. By adding a known quantity of atropine to a sample of the drug and carrying out the modified procedure, it was shown that no loss of alkaloid occurred during the digestion.

Results comparable to those of the official method may be obtained with a material improvement in the procedure from the standpoint of decreasing the time and equipment involved and the facility of operation.

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The Antibacterial Properties of Cyanine*

By DONALD W. McKINSTRY†

The bacteriostatic effect of cyanine (quinoline blue) on a variety of pathogenic microorganisms *in vitro* was determined. The data obtained indicate that this compound is remarkably effective against *Corynebacterium diphtheriae* and the Gram-positive cocci. The amount of drug tolerated by Swiss albino mice compared favorably with that of several other medicaments employed today in therapy of certain infectious diseases. The therapeutic possibilities of this compound in human and veterinary medicine are discussed.

THE MARKED antibacterial action of cyanine (1,1′-di-isoamyl-4,4′-quinocyanine iodide, $C_{29}H_{35}N_2I$) was noted during a recent survey of synthetic organic dyes for antibacteriophage activity (1). Although the compound was found to be devoid of any significant antiviral properties, its extraordinary bacteriostatic effect against *Staphylococcus aureus* prompted an investigation of its activity in this respect with other pathogenic microorganisms.

The first biologic property of cyanine was discovered by Paul Ehrlich (2) in 1887 when he succeeded in staining central nervous tissue *in vivo* with an alcoholic solution of the dye mixed with serum. Behring (3), two years later, found that the dye was a good bactericide but did not investigate its selective antibacterial properties. Tagami (4) confirmed Behring's discovery and also noted that its bactericidal action was not influenced by the *p*H of the medium. Tappeiner

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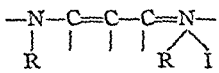
and Jodlbauer (5) discovered that the compound was endowed with protozoacidal properties and Martinotti (6) found it to be very toxic for tadpoles.

Browning and his co-workers (7) in 1926 began an intensive systematic study of the antiseptic action of the quinoline dyes in an effort to determine the relationship between chemical structure and bactericidal properties. They found that the structure,



present in cyanine and acriflavine, in which a trivalent, basic nitrogen atom is joined to a pentavalent nitrogen atom by a carbon chain in which there are alternating double bonds, appeared to be essential for bactericidal action. *Staphylococcus aureus* and *Escherichia coli* were employed as test organisms in this study. Although the results obtained by these workers amply confirmed the previously published reports concerning the remarkable bactericidal action of cyanine, the compound was not investigated with regard to its application as a therapeutic agent in microbiological diseases of man and domesticated animals. During the course of this investigation Brooker and Sweet (8) reported the effect of a series of cyanine, styryl and related dyes on certain bacteria, parasites, protozoa and viruses. One of the compounds studied—(1-amyl-2,5-dimethyl-3-pyrrole)(1,6-dimethyl-2-quinoline) di-methinecyanine chloride—inhibited the growth of *Lactobacillus casei*, *L. arabinosus*, *Staphylococcus fecalis*, and *E. coli in vitro*. None of the compounds was therapeutically effective against a number of infectious agents *in vivo*. Cyanine is employed primarily today for the isochromatization of photographic plates and to a limited extent by histologists for staining fats, bone tissue, and lignified membranes.

This paper describes the bacteriostatic effect of cyanine on a variety of pathogenic microorganisms *in vitro* and its toxicity for experimental animals.

MATERIALS AND METHODS

Test Microorganisms.—A variety of pathogenic microorganisms, including Gram-positive cocci, Gram-negative cocci, Gram-positive spore-forming rods, and Gram-negative nonspore-forming rods, were employed in this study in an effort to determine the microbiological limits of the antibacterial action of the drug.

Media.—A standard medium of a constitution known to satisfy the nutritional requirements for optimal growth and activity was used for each microorganism.

Inoculum.—In order to make the assay as rigorous and critical as possible, a massive inoculum consisting of 0.1 ml. of a vigorous eighteen- to forty-eight-hour broth culture was used to inoculate each tube of drug-treated broth as well as the culture control.

Antibacterial Assay.—Serial dilutions of the drug were prepared in the medium of choice for the organism under investigation. Each tube of drug-treated medium was then inoculated as described above. Adequate culture controls and drug controls were included in each assay. The cultures were incubated for from forty-eight to ninety-six hours at the temperature optimal for growth of the organism in question. All culture tubes were carefully examined and the extent of bacterial growth determined under standard conditions at the end of the incubation period.

Criterion of Antibacterial Potency.—The degree of inhibition of growth as evidenced by the concentration of bacterial cells in the drug-treated medium as compared with that of the untreated bacterial control was used as the criterion of antibacterial potency. The concentration of bacterial cells in each culture was determined by turbidimetric methods using the transillumination technique with the aid of a comparator box and a standard light source. The bacteriostatic end point was considered to be the highest dilution of drug that completely inhibited growth of the microorganism studied.

Acute Toxicity for Mice.—In an effort to determine whether the toxic effects of the compound were too great to warrant exploration of its possibilities as a therapeutic agent for infectious diseases, the acute toxicity for mice was determined. Graded doses of the drug in physiologic saline were administered intraperitoneally to young Swiss albino mice. Ten mice were employed in each group for a given dosage. The animals were observed for a period of seven days and examined daily for evidence of toxic manifestations. The LD_{50} dose of drug for this experimental animal was determined by the method of Reed and Muench (9).

EXPERIMENTAL RESULTS

The bacteriostatic effect of cyanine against the microorganisms employed in this study is summarized in Table I.

The toxicity of the drug in terms of the LD_{50} dose for young Swiss albino mice is presented in Table II.

DISCUSSION

Examination of the bacteriostatic assay data presented in Table I reveals that cyanine is remarkably effective *in vitro* against a variety of pathogenic microorganisms. Its deleterious effect in minute concentration on *Corynebacterium diphtheriae* and the pathogenic Gram-positive cocci is especially noteworthy. The extraordinary susceptibility of *S. aureus* to this drug coupled with its strong affinity for osseous tissue indicates that its possible therapeutic application in staphylococcal osteomyelitis merits investigation.

The amount of cyanine tolerated by the mouse (Table II) compares favorably with that of several

TABLE I.—ANTI-BACTERIAL ACTION OF CYANINE

Microorganism[a]	Medium	Growth of Microorganism in Indicated Dilution of Drug												Culture Control	Bacteriostatic Titer
		10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	10^{11}	10^{12}		
Staph. aureus (PJ1B)	Nutrient broth	–	–	–	–	–	–	–	–	–	±	++	++++	++++	10^0
Strep hemolyticus (C-203)	Veal infusion broth	–	–	–	–	–	–	±	+	+++	++++	++++	++++	++++	10^0
Strep. agalactae	Veal infusion broth	–	–	–	–	–	±	+	++	+++	++++	++++	++++	++++	10^5
Strep. dysgalactae	Veal infusion broth	–	–	–	–	–	±	+	+++	++++	++++	++++	++++	++++	10^5
Strep. uberis	Veal infusion broth	–	–	–	–	–	–	+	+	++	++++	++++	++++	++++	10^6
Diplococcus pneumoniae	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^5
Neisseria intracellularis	Veal infusion Destrose broth	–	–	–	–	–	–	–	±	+	+	+++	++++	++++	10^7
Eberth. typhosa (P-63)	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^5
Sal. paratyphi	Veal infusion broth	–	–	–	–	–	–	+++	++++	++++	++++	++++	++++	++++	10^6
Sal. schott-müelleri	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^5
Sal. enteritidis	Veal infusion broth	–	–	–	–	–	±	++	++++	++++	++++	++++	++++	++++	10^5
Sal. gallinarum	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^5
Sal. typhimurium	Veal infusion broth	–	–	–	–	–	++	++++	++++	++++	++++	++++	++++	++++	10^5
Shig dysenteriae (K-7C)	Veal infusion broth	–	–	–	–	–	±	+++	++++	++++	++++	++++	++++	++++	10^5
Shig. dysenteriae (Sonnei)	Veal infusion broth	–	–	–	–	–	+++	++++	++++	++++	++++	++++	++++	++++	10^5
Coryne. diphtheriae	Veal infusion ascitic fluid medium	–	–	–	–	–	–	–	±	++	++++	++++	++++	++++	10^7
Klebsiella. pneumoniae	Veal infusion broth	–	+	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	10^1
Pseudomonas aeruginosa	Nutrient broth	–	–	–	–	+	++	++++	++++	++++	++++	++++	++++	++++	10^4
Clostridium. welchii	Brewer's medium	–	–	–	–	–	+++	++++	++++	++++	++++	++++	++++	++++	10^5

[a] The results obtained with *Neisseria gonorrheae* and *mycobacterium tuberculosis* (human strain) were inconclusive.

TABLE II.—ACUTE TOXICITY OF CYANINE FOR MICE

Dilution of Compound	Inoculum, Ml.	Dose, Mg.	Route Adm.	Number of Mice	Weight of Mice, Gm.	Death of Mice (Days) 1	2	3	4	5	6	7	Mortality, %	LD_{50} Mg./Kg.
10^2	0.1	1	IP[a]	10	20	9	1	0	0	0	0	0	100	
10^3	0.1	0.1	IP	10	20	0	0	0	0	0	0	0	0	
10^4	0.1	0.01	IP	10	20	0	0	0	0	0	0	0	0	16
10^5	0.1	0.001	IP	10	20	0	0	0	0	0	0	0	0	

[a] IP, intraperitoneally.

other medicaments employed today in the chemotherapy of certain infectious diseases. The history of chemotherapy is prominently marked by the discouraging cases where compounds discovered to be singularly effective against a particular infectious agent were later found to be too toxic for the host when tested against the infectious process *in vivo*. Fortunately, the limited toxicity of cyanine does not preclude exploration of its therapeutic possibilities in human and veterinary medicine.

Further studies of the effect of this compound in experimental bacterial infections is planned and will be reported in a later publication.

SUMMARY AND CONCLUSIONS

1. The bacteriostatic effect of cyanine (quinoline blue) on a variety of pathogenic microorganisms is described.

2. The toxicity of the drug in terms of the LD_{50} dose for young Swiss albino mice is given.

3. The possible application of the compound in the therapy of certain microbiological diseases is discussed.

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Experiments on the Constitution of Umbellatine*

By R. CHATTERJEE†

Based on the experimental work described in this report, the correct formula for umbellatine is believed to be $C_{21}H_{23}O_6N$. The alkaloid has been found to contain one methylenedioxy, two methoxyl and four hydroxyl groups. A structural formula (VII) is suggested for umbellatine

THE ALKALOID UMBELLATINE, $C_{21}H_{23}O_6N$, has been isolated from several Indian species of *Berberis* (1) and *Mahonia* (2). It has been used with success in the treatment of oriental sore. The pharmacological action of umbellatine is similar to that of berberine but more intense, except in cardiovascular responses which are less than those caused by berberine (3).

It has been shown that umbellatine is a tertiary base. It contains one methylenedioxy, two methoxyl, and four hydroxyl groups (4). On heating with zinc dust in a current of hydrogen, *iso*quinoline is obtained, which shows that the nitrogen atom together with its methyl group is attached to a ring and that it belongs definitely to the *iso*quinoline group of alkaloids. From a comparison of the ultraviolet absorption curve of umbellatine with that of berberine, it has been concluded that 2:3-methylenedioxytetrahydro*iso*quinoline ring forms a part of the umbellatine nulceus (4). Moreover, to degrade umbellatine into a nitrogen-free compound, Hofmann degradation has to be repeated twice for the nitrogen valences are involved in a hydrogenated ring system.

The Linking of the Benzene Nucleus to the Isoquinoline Nucleus.—Umbellatine gives *ortho*hemipinic acid on oxidation with potassium permanganate (5) and opianic acid with manganese dioxide and sulfuric acid. In both cases of oxidation, nitrogenous fragment of the

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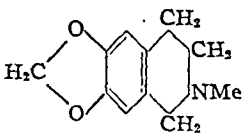
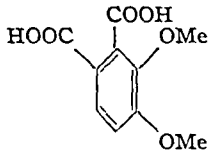
† The author expresses his thanks to the University of Calcutta for a Sir P. C. Ray Research Fellowship.

umbellatine molecule cannot be isolated. The *iso*quinoline part of the molecule may be weaker due to the presence of substituents like phenolic hydroxyl groups.

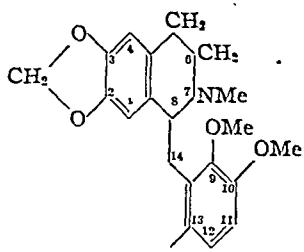
The evidence for the presence of a methylenedioxytetrahydro*iso*quinoline skeleton (4) and a hemipinic or opianic acid nucleus gives a fair indication to the structure of umbellatine.





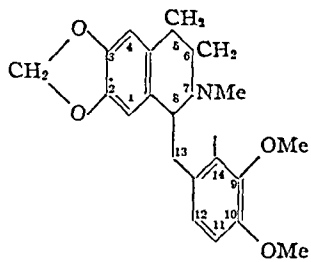
If the linking of the dimethoxybenzene portion to the tetrahydro*iso*quinoline nucleus is assumed to be of benzyl*iso*quinoline type, where the dimethoxybenzene portion is linked to the *iso*quinoline part by a methylene group, the easily breakable point will be at the methylene group producing a carboxyl or an aldehyde group. For instance, papaverine produced on alkali fusion, veratric acid (6), and laudanosine, on oxidation with manganese dioxide and sulfuric acid, yielded veratraldehyde (7). Here the production of opianic acid from umbellatine indicates a benzyl*iso*quinoline structure with a substituent in the *ortho* position to the linkage, which can be oxidized to an aldehyde group. Another suggestion would be the hydrastine structure, for both hydrastine (8) and narcotine (9) yielded opianic acid on oxidation. Since umbellatine has not been found to undergo hydrolytic decomposition, the lactonic group is, in all probability, absent from it. The corydaline structure (10) is not applicable to it, for a methyl group is already attached to the nitrogen atom in it. The nitrogen atom common to two rings, with a group attached to it, has to be pentavalent and, as such, the formation of salts of umbellatine of the type B, HX with acids cannot be explained. A base like berberine or palmatine (10) with a hydroxyl group attached to the nitrogen atom, where the latter is pentavalent, only forms salts by the elimination of water. The skeleton of umbellatine is represented by (I) or (II), the benzyltetrahydro*iso*quinoline structure with a substituent in position 13 or 14 which can be oxidized to a carboxyl or an aldehyde group.

Discussion of the Position of the Hydroxyl Groups.—The positions 2 and 3 are occupied by the methylenedioxy group; 9 and 10 by the two methoxyl groups. Four of the positions 1, 4, 5, 6, 8, 13, and 14 are occupied by the four hydroxyl groups. The aldehyde group of the opianic acid is developed in position 13



4 OH groups

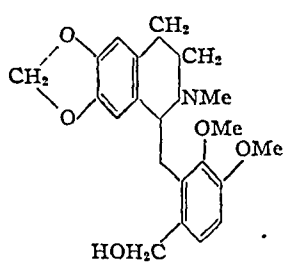
(I)



4 OH groups

(II)

of formula (I), after fission of the linkage at 14. It is very likely obtained by the oxidation of a primary alcoholic group placed at that position in the base. The structure of umbellatine is, therefore, preferably represented by the skeletal formula (I), with a primary alcoholic group definitely in position 13, for the location of this group in 14 in formula (II) cannot explain the production of

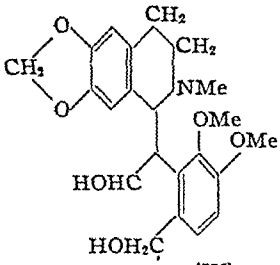


3 OH groups

(III)

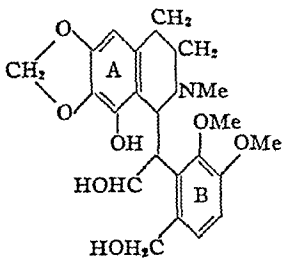
opianic acid. The skeletal formula of umbellatine can be written as (III). In hydrastine and narcotine the linking at 14 is by a latent :CHOH group. Considering a similar possibility of a potential hydroxyl group in 14 (II), the structural formula of umbellatine can be extended further to (IV). One of the two remaining hydroxyl groups is positively phenolic in character. Either of the positions 1 and 4 can only be allotted to it, when umbellatine will be represented either by (V) or (VI).

There remains now to locate the position of only one hydroxyl group, probably one of the two hydroxyl groups, which can be acetylated.



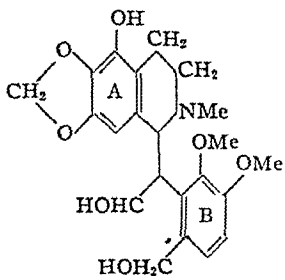
2 OH groups

(IV)



1 OH group

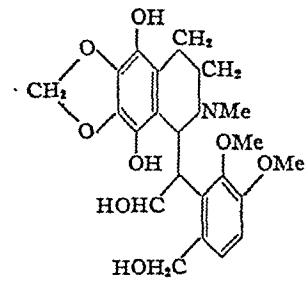
(V)



1 OH group

(VI)

The phenolic character of this hydroxyl group can only be inferred from a slight hyperchromic effect in the absorption spectra and the *p*H value (5.7) of its 0.5 per cent aqueous solution. Unfortunately in cases of oxidation by potassium permanganate, manganese dioxide and sulfuric acid, chromic acid and sulfuric acid, chromic acid and acetic acid, the nitrogenous fragment or a product containing the methylenedioxy group cannot be isolated. This shows that the ring A containing the methylenedioxy group is not as stable to oxidation as ring B, containing the two methoxyl groups. The instability may be due to the presence of phenolic hydroxyl group or groups in ring A. Methyl umbellatine is oxidized by different agents, but neither a product containing ring A nor a nitrogenous fragment can be isolated. Had there been only one phenolic group in position 1 or 4, and if this group were methylated before oxidation, a fragment like cotarnic acid would be very likely to be obtained. So the instability of the ring A in both cases of umbellatine and its methyl derivative may well be due to the phenolic hydroxyl groups both in positions 1 and 4. Positions 5 and 6 are not considered because alkaloids have not been isolated in which these positions carry a substituent (11). A hydroxyl group cannot possibly be placed at position 8, since two hydroxyl groups are not very likely present in adjacent positions, and no alkaloid of benzyltetrahydro-*iso*quinoline structure is yet known with hydroxyl group at 8. The structural formula of umbellatine is finally suggested to be (VII).



(VII)

EXPERIMENTAL

Zinc Dust Distillation.—Umbellatine (2 Gm.) was mixed with zinc dust (20 Gm.) and heated strongly in a combustion tube over a free flame, the air in the tube being replaced by a current of hydrogen. The reaction mass was kept stirred by constant rotation of the tube, which was heated to redness within ten minutes. The reaction product which was a brownish liquid was taken in dilute hydrochloric acid (5%) and the mixture was slightly warmed. The residue in the tube was cooled and then digested three times with ether (50 cc. each time). The brown ether solution was shaken with the above hydrochloric acid solution. The ether layer was treated separately. The ether was removed, and the residue, a tarry product, could not be successfully worked up. The acid solution was then made alkaline with dilute sodium hydroxide solution (5%) and the mixture was repeatedly extracted with ether. The ether layer was washed with water and then dried over anhydrous sodium sulfate. The residue obtained, after removal of ether, was subjected to a high vacuum distillation, when a drop of a colorless liquid was obtained at 70°/0.3 mm. pressure. The liquid gradually became yellow in air. It was identified as *iso*quinoline by the preparation of the following derivatives.

Isoquinoline Picrate.—A part of the liquid was taken in dry ether and mixed with a solution of picric acid in ether. It was kept for an hour in a refrigerator until a yellow-colored picrate separated. The crude picrate melted at 195°. The picrate was difficultly soluble in alcohol from which it was repeatedly crystallized. The pure product was obtained as pale yellow needles which vaporized to a certain extent at 200° and finally melted sharply at 215°. The picrate of a synthetic specimen of *iso*quinoline melted similarly at 215° after partial

vaporization at 200°. The m. p. did not change when the two samples were mixed together.

Anal.—Calcd. for $C_9H_7N \cdot C_6H_2N_3O_6$: N, 15.3. Found: 15.5.

Isoquinoline Chloroplatinate.—The liquid obtained by high vacuum distillation was mixed with dilute hydrochloric acid and treated with a solution of chloroplatinic acid (5%) when a pale orange precipitate separated. On warming the mixture, the precipitate went in solution, from which pale orange-colored crystals separated on cooling. The chloroplatinate which was recrystallized from water shrank at 258° and melted with decomposition at 260–262°. An authentic specimen of *iso*quinoline chloroplatinate had the same m. p. and when both samples were mixed, the m. p. was not depressed.

Anal.—Calcd. for $(C_9H_7N)_2H_2PtCl_6$: Pt, 28.2. Found: 27.9.

Hofmann Degradation

Iodomethylate of Methyl Umbellatine.—Methyl umbellatine was dissolved in methyl alcohol and treated with excess of methyl iodide. On leaving overnight brilliant yellow crystals of the methiodide were obtained which decomposed without melting.

Anal.—Calcd. for $C_{23}H_{30}O_8NI$: I, 22. Found: 19.8.

Methine of Methyl Umbellatine.—Iodomethylate of methyl umbellatine (4.0 Gm.) was placed in boiling water (200 cc.) and refluxed with potassium hydroxide (45 Gm.) in methyl alcohol (25 cc.) for six hours, when most of the substance decomposed and the methine was obtained as a thick, viscous brown mass. The brown mass was boiled for some time to get rid of the methyl alcohol, and then extracted with ether. The ether extract could not be solidified.

Iodomethylate of the Methine.—The crude methine was dissolved in methyl alcohol and then treated with excess of methyl iodide. The methiodide was crystallized from methyl alcohol as yellow needles, which decomposed above 200°.

Anal.—Calcd. for $C_{23}H_{30}O_8NI$: C, 72.7; H, 7.9; I, 33.4. Found: C, 72.0; H, 8.1; I, 33.0.

Decomposition of the Iodomethylate of the Methine.—When an alcoholic solution of the iodomethylate was refluxed with potassium hydroxide solution for eight hours, trimethylamine was obtained. The vinyl derivative thus obtained forms a heavy oil, which was taken up in chloroform. The extract after removal of chloroform could not be solidified.

Oxidation of the Iodomethylate of the Methine.—The iodomethylate (1.0 Gm.) in water (100 cc.) was treated with potassium permanganate solution (1%) at the ordinary temperature, until the color of the solution remained pink. The mixture was then warmed on a water bath, to complete the oxidation, which required further addition of the permanganate solution until pink. The filtered solution was decolorized by sulfur dioxide and then concentrated *in vacuo* to about 100 cc. A large amount of inorganic matter was filtered off. The solution was then acidified with hydrochloric acid and continuously extracted with ether. The ether was evaporated and the pasty mass of the residue was distilled *in vacuo*, when colorless needles were obtained at 150–160°/0.1 mm. The needles on crystallization from water were eventually identifie to be hemipinic acid, obtained previously by th permanganate oxidation of the base umbellatin itself.

Oxidation of the Vinyl Compound.—The vin compound obtained by the decomposition of th iodomethylate of the methine was oxidized by th method described above, to obtain finally crystals c hemipinic acid.

Oxidation Experiments

Manganese Dioxide and Sulfuric Acid.—Um bellatine (2.5 Gm.) was dissolved in a mixture o sulfuric acid (5 Gm.) and water (200 cc.) an mechanically stirred with manganese dioxide (Gm.) for an hour on a water bath. The mixtur slowly became brown and turbid. The reactio was rather lazy and the heating was continued fo another two hours. After cooling the mixture wa filtered. The brown-colored granular residue wa dried in air and extracted with ether in a Soxhlet apparatus. On removal of ether no residue wa left behind. The filtrate was extracted exhaustively with ether. The ether was removed and the residue was sublimed as a white crystalline substance a 160°/0.1 mm. The aqueous solution was then mad ammoniacal and exhausted with ether. The residue obtained, on removal of ether, was sticky and could not be worked up successfully.

Identification of Opianic Acid.—The white crystalline substance, thus obtained was crystallized from water as fine colorless prisms; m. p. 147°. It did not depress the m. p. of opianic acid obtained by the oxidation of narcotine by concentrated sulfuric acid and manganese dioxide (12). The opianic acid obtained from narcotine melted at 150°.

Anal.—Calcd. for $C_{10}H_{10}O_5$: C, 57.1; H, 4.8. Found: C, 57.1; H, 4.6.

Oxime of Opianic Acid.—Opianic acid (1 mol.) was dissolved in a dilute solution of potassium hydroxide (4 mols.), mixed with hydroxylamine hydrochloride (1.5 mols.), and left overnight. The mixture was then acidified with hydrochloric acid and allowed to stand, when needle-shaped crystals separated which were collected and crystallized twice from warm water; m. p. 82°.

Anal.—Calcd. for $C_{10}H_{11}NO_5$: N, 6.2. Found: N, 6.1.

Potassium Permanganate.—Umbellatine hydrochloride (5 Gm.) was dissolved in warm water (150 cc.) and treated with potassium carbonate (1.0 Gm.) solution and then oxidized by slowly running in a solution of potassium permanganate (5%). The decolorization of the permanganate was very rapid at the start, and as soon as about 330 cc. of the solution was added decolorization slowed down appreciably and continued warming was necessary to remove the last traces of the pink color of the permanganate solution.

The solution was left overnight and then saturated with carbon dioxide gas and filtered from the manganese precipitate. The filtrate and the washings were evaporated completely to dryness on a water bath, and the residue was soxhletted with absolute alcohol. The residue obtained after the removal of the last traces of alcohol was dissolved in water and the brown aqueous solution was treated with diluted sulfuric acid when a small quantity of sticky matter separated which could not be solidified. The sticky

matter was removed by filtration and the filtrate was extracted continuously with ether (100 cc. in four installments) by shaking each time in a mechanical shaker for eight hours. The ethereal solution on removal of ether yielded a pale yellow oily residue which solidified when kept in a vacuum desiccator over concentrated sulfuric acid for a week. It was then sublimed at 0.1 mm. pressure when a minute quantity of a yellow liquid separated first at 140–150°, and then a transparent crystalline solid, moist with the liquid at 150–160°. The moist solid when dried on a porous plate melted at 125–157°.

The moist solid was well washed with small quantities of ether, dried and powdered and warmed with concentrated hydrochloric acid and kept overnight and filtered from the acid solution and repeatedly washed with cold water in small amounts. The filtrate and the washings yielded a very small quantity of a semisolid which could not be successfully handled. The residue was then dried; m. p. 155–160°. The m. p. further rose to 177–178.5° on slow crystallization of the solid from boiling water.

Anal.—Calcd. for $C_{10}H_{10}O_6$: C, 53.1; H, 4.43. Found: C, 52.96; H, 4.75.

Hemipinic Acid.—To identify the hemipinic acid thus obtained, the m. p. of the sample was compared with that of hemipinic acid prepared from opianic acid, which in turn was obtained from narcotine (13). The hemipinic acid as obtained from narcotine melted at 179–180° and, when mixed with that obtained from umbellatine, melted at 179°.

Ethylimide of Hemipinic Acid.—The ethylimide derivative of hemipinic acid was prepared according to Liebermann (14) by dissolving the purified acid obtained from umbellatine in an aqueous solution of ethylamine (30%) and evaporating it to dryness. The residue thus obtained was heated carefully over a naked flame, and then exhausted with boiling methyl alcohol. On cooling the ethylimide separated as needles; m. p. 90°. The m. p. did not change even on high vacuum sublimation of the ethylimide at 125–135°/0.15 mm. pressure. The ethylimide of hemipinic acid, obtained from narcotine, was purified by the author by high vacuum sublimation and by crystallization from water, and was found to melt sharply at 92°. This m. p. was not depressed by the ethylimide of hemipinic acid obtained from umbellatine. This shows that the hemipinic acid thus obtained is 3,4 dimethoxy phthalic acid.

Anal.—Calcd. for $C_{10}H_9O_4N$: N, 5.9. Found: N, 5.63.

Chromic Acid and Sulfuric Acid.—The base (2 Gm.) was dissolved in water (100 cc.) and warmed on a water bath with a mixture of chromic acid (1.0 Gm.) and concentrated sulfuric acid (1.4 Gm.) and water (20 cc.). After one hour an equal quantity and, after another half hour, half the quantity of chromic acid were added. After two hours when no more chromic acid was being used up, the excess was reduced by sulfur dioxide. The mixture was filtered and extracted exhaustively with ether.

Treatment of the Ether Layer.—On removal of ether, a small quantity of sticky substance remained which, on vacuum sublimation, yielded colorless needles at 130°/0.08 mm. The needles, on crystallization from boiling water, melted at 177°, and eventually were identified to be hemipinic acid, through the ethylimide of the acid.

Treatment of the Aqueous Layer.—The green-colored aqueous solution was nearly neutralized by barium hydroxide solution. The precipitate was filtered and boiled with water. The remaining sulfuric acid was removed from the combined filtrate by barium carbonate. The neutral solution thus obtained, was evaporated, and the residue extracted with alcohol. The residue left, on removal of alcohol, was dissolved in water, and heated carefully with ammonium hydroxide; no solid separated. The ammoniacal solution was then extracted with water. The ether extract was distilled in high vacuum, and neither a solid nor a liquid separated.

The Molecular Formula of Umbellatine.—The molecular formula of umbellatine was reported to be $C_{21}H_{21}O_8N$, on the basis of combustion analyses of the base and its salts. Now a study of its degradation products has led to changing the formula to $C_{21}H_{25}O_8N$, without any appreciable change in its percentage composition. Found: C, 59.0, 59.2; H, 5.33, 5.4; N, 3.3, 3.3; OMe, 14.4, 14.4. $C_{21}H_{25}O_8N$, $^1/_2H_2O$ requires C, 59.2; H, 5.6; N, 3.3; OMe, 14.6. $C_{21}H_{21}O_8N$, $^1/_2H_2O$ requires C, 59.4; H, 5.2; N, 3.3; OMe, 14.6%. Pt, 15.68, 15.71.

Anal.—Calcd. for $(C_{21}H_{25}O_8N)_2$, H_2PtCl_6; Pt, 15.73. Found: Pt, 15.72%.

SUMMARY

1. Umbellatine is an alkaloid obtained from some of the Indian species of *Berberis* and *Mahonia*. Its formula is correctly represented by $C_{21}H_{25}O_8N$.

2. Umbellatine is a tertiary base, and it contains one methylenedioxy, two methoxyl, and four hydroxyl groups.

3. On oxidation with manganese dioxide and sulfuric acid, it produces opianic acid; with potassium permanganate or chromic acid and sulfuric acid, *ortho*hemipinic acid is obtained.

4. On zinc dust distillation, it gives *iso*quinoline.

5. The structural formula of umbellatine is suggested to be (VII).

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The Synthesis of Some Esters of *p*-Carboxybenzamidine*

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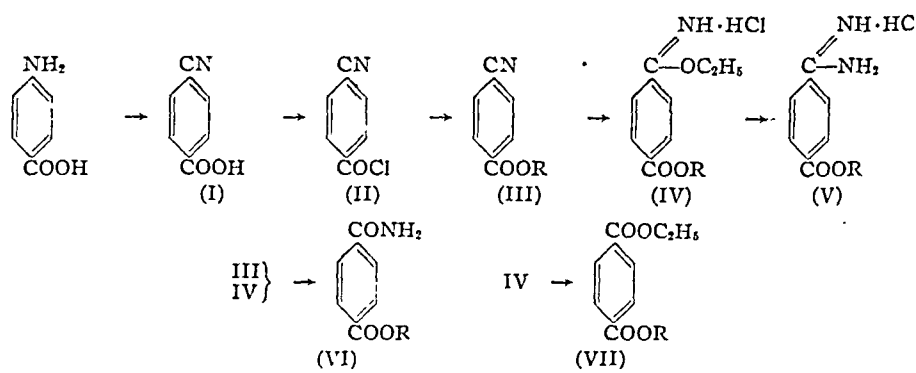
An attempt was made to synthesize some esters of *p*-carboxybenzamidine wherein their structure resembled that of the common local anesthetics in general use. The compounds synthesized differ from the esters of *p*-aminobenzoic acid only in that the amidino group replaces the *p*-amino group. The methyl through hexyl series of esters of *p*-carboxybenzamidine were prepared.

THE OBJECT of this investigation was to prepare some esters of *p*-carboxybenzamidine wherein their structure resembled that of the common local anesthetics of the *p*-aminobenzoic acid type. The compounds synthesized differ from the esters of *p*-aminobenzoic acid only in that the amidino group replaces the *p*-amino group. The study was undertaken to try to improve the general method for the preparation of unsubstituted amidines, particularly those containing an ester grouping in the molecule. The chemical properties of the imido ester hydrochloride (1–4), an intermediate, led to the preparation of some mixed esters of terephthalic acid and some esters of terephthalamic acid.



R = H, CH_3, C_2H_5, C_3H_7, n-C_4H_9, n-C_5H_{11}, or n-C_6H_{13}.

EXPERIMENTAL

p-Cyanobenzoic Acid (I).—An alkaline solution of cuprous cyanide was prepared from the cuprous chloride, which was obtained from 0.6 *M* of copper sulfate, 0.625 *M* of sodium chloride, 0.3 *M* of sodium bisulfite, and 0.51 *M* of sodium hydroxide, and 1.25 *M* of sodium cyanide in 200 cc. of water to which was added 0.5 *M* of monohydrated sodium carbonate. To this solution was added a diazonium solution prepared from 1 *M* of hydrochloric acid, 0.5 *M* of *p*-aminobenzoic acid, and 0.5 *M* of sodium nitrite. The diazonium solution was maintained at 0–5°, while it was slowly added to the alkaline cuprous cyanide solution with stirring. The resulting mixture was heated at 60° for thirty minutes to drive off the nitrogen and the carbon dioxide that formed. The alkaline reaction mixture was cooled in a refrigerator for several days to allow a large mass of crystalline sodium *p*-cyanobenzoate to form. This crystalline mass represents about 50% of the total yield obtained. The mother liquor obtained after filtration was acidified with hydrochloric acid and filtered to collect the precipitated crude *p*-cyanobenzoic acid which was then treated with ammonium hydroxide to dissolve out the *p*-cyanobenzoic acid as the ammonium salt. The insoluble inorganic copper salts were filtered off. The filtrate was acidified and chilled and filtered to obtain an impure *p*-cyanobenzoic acid. The impure product was purified after several recrystallizations from hot water and decolorized with charcoal. The sodium salt of the acid obtained previously was dissolved in water, the solution was acidified, and the precipitated acid recrystallized from water. This modification in the method of preparation of the acid produced a yield of 68%. Melting point found 219°, reported 219° (5).

p-Cyanobenzoyl Chloride (II).—To one mole of *p*-cyanobenzoic acid in a one-liter, round-bottom flask equipped with a reflux condenser and a calcium chloride drying tube, was added 291 cc. of thionyl

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chloride. The mixture was heated on a steam bath for one hour. The excess thionyl chloride was then removed with heat under vacuum of a water pump. The residual oil was poured over crushed ice. The crystalline acid chloride was filtered off, dried, and recrystallized from petroleum ether. (When smaller reaction quantities are used the excess thionyl chloride may be allowed to evaporate off spontaneously in a well-ventilated hood and the crude acid chloride then recrystallized from petroleum ether.) The total yield was 90%. The melting point found 65°, reported 65° (6).

p-Cyanobenzoic Acid Esters (III).—To 0.2 *M* of *p*-cyanobenzoyl chloride was added 30 cc. of the appropriate alcohol in small portions. The mixture was allowed to stand for a short time when an evolution of heat occurred and gaseous hydrogen chloride formed. Water and sodium bicarbonate were added to the reaction mixture and then the neutralized mixture was chilled and filtered. The precipitated ester was then recrystallized from alcohol and water.

β-Diethylaminoethyl Ester of *p*-Cyanobenzoic Acid Hydrochloride.—To 0.2 *M* of *p*-cyanobenzoyl chloride in 150 cc. of benzene was added slowly a solution of 0.188 *M* of β-diethylaminoethyl alcohol in 100 cc. of benzene. The reaction mixture was heated for one hour under a reflux condenser over a steam bath. The crude ester which had separated was collected on a filter and dried. It was purified by recrystallization from absolute alcohol. The mother liquor was diluted with ether for a further yield of product. The total yield was 84.7%. Melting point was 159–160°.

Anal.—Calcd. for $C_{14}H_{19}O_2N_2Cl$: C, 59.46; H, 6.77. Found: C, 59.47; H, 6.99.

p-Carbethoxyimidobenzoic Acid Ester Hydrochlorides (IV).—One-tenth mole of the *p*-cyanobenzoic acid ester, 100 cc. of anhydrous ether, and 10 cc. of absolute alcohol were introduced into a 250-cc. flask equipped with a two-hole rubber stopper for a gas inlet tube and a calcium chloride drying tube and the mixture was weighed and chilled to 0°. Anhydrous hydrogen chloride was passed into the solution at a moderate rate. After thirty minutes the gas inflow was stopped and the flask weighed. Between 21 and 27 Gm. of gas was absorbed in this

TABLE I.—ESTERS OF *p*-CYANOBENZOIC ACID

Ester	Yield, %	Melting Point, ° C. Found	Melting Point, ° C. Reported	Formula	Analysis, % C and H Found	Analysis, % C and H Calcd.
Methyl	75	62	62	$C_9H_7O_2N$	.....	.....
Ethyl	85.8	54	54	$C_{10}H_9O_2N$	.....	.....
Propyl	68.8	40–41	..	$C_{11}H_{11}O_2N$	C, 69.90 H, 6.01	C, 69.82 H, 5.86
n-Butyl	77	56–57	..	$C_{12}H_{13}O_2N$	C, 70.91 H, 6.51	C, 70.92 H, 6.45
n-Amyl[a]	63.5	18–19	..	$C_{13}H_{15}O_2N$	C, 71.59 H, 7.09	C, 71.86 H, 6.96
n-Hexyl	63	45–46	..	$C_{14}H_{17}O_2N$	C, 72.93 H, 7.74	C, 72.69 H, 7.41

[a] The amyl ester was an oil at room temperature and was purified by distillation under vacuum.

TABLE II.—*p*-CARBETHOXYIMIDOBENZOIC ACID ESTER HYDROCHLORIDES

Ester	Yield, %	Melting Point, ° C. Found	Melting Point, ° C. Reported	As Free Base[a] Melting Point, ° C. Found	As Free Base[a] Melting Point, ° C. Reported
Methyl	77	202–203	.....	Oil	...
Ethyl	70	173–174	178–179	73–74	Oil
Propyl	68	114–115	.....	Oil	...
n-Butyl	81	74–76	.....	Oil	...
n-Amyl	70	92–94	.....	Oil	...
n-Hexyl	70	101–102	.....	Oil	...

[a] These were prepared by direct neutralization with a 5% sodium bicarbonate solution and extracted with ether. The oils were reported as such at room temperature.

TABLE III.—ESTERS OF *p*-CARBOXYBENZAMIDINE HYDROCHLORIDE

Ester	Yield, % Method 1	Yield, % Method 2	Melting Point, ° C.	Empirical Formula	Analysis % C and H Found	Analysis % C and H Calc.
Methyl	78.5[b]	85.2[g]	326–327 Over 250 (S)	$C_9H_{11}O_2N_2Cl$	C, 50.83 H, 5.40	C, 50.37 H, 5.16
Ethyl	89[c]	84[h]	207–208 found 217–218 rep.	$C_{10}H_{13}O_2N_2Cl$	C, 52.67 H, 5.83	C, 52.52 H, 5.73
Propyl	63[d]	75[i]	170–171.5	$C_{11}H_{15}O_2N_2Cl$	C, 54.77 H, 6.52	C, 54.43 H, 6.23
n-Butyl	84[e]	75[j]	149–150	$C_{12}H_{17}O_2N_2Cl$	C, 56.49 H, 7.23	C, 56.12 H, 6.76
n-Amyl	70[f]	89[k]	141–142	$C_{13}H_{19}O_2N_2Cl$	C, 57.68 H, 7.14	C, 57.66 H, 7.07
n-Hexyl	87[a,m]	85[l]	139–141	$C_{14}H_{21}O_2N_2Cl$	C, 59.63 H, 7.84	C, 59.04 H, 7.46

[a] Ammonium sulfate replaced ammonium chloride since the ammonium chloride reaction produced the amidine hydrochloride which was low melting and had water of hydration.

[b–m] The amounts of imido ester hydrochlorides reacted were as follows: *b*, 8.3 Gm.; *g*, 4.0 Gm.; *c*, 9.75 Gm.; *h*, 10 Gm.; *d*, 7.0 Gm.; *i*, 2.3 Gm.; *e*, 10 Gm.; *j*, 7.0 Gm.; *f*, 10 Gm.; *k*, 4.6 Gm.; *m*, 5.0 Gm.; *l*, 3.0 Gm.

time. The flask was stoppered and set in the refrigerator for several to ten days. During this time the imido ester hydrochloride crystals were filtered off and the mother liquor returned to the refrigerator for additional yields. The exhausted mother liquor was then treated to obtain the unreacted ester by removing the excess gas and evaporating off the ether and recrystallizing the ester from alcohol and water.

p-Carboxybenzamidine Esters (V).—The initial steps in *Method 1* (7, 8) were the neutralization of the ester of *p*-carbethoxyimidobenzoic acid hydrochloride with a 5% solution of sodium bicarbonate (9) and the extraction with ether in a separatory funnel. The ether extracts were combined and dried over anhydrous sodium sulfate. The ether was then evaporated off and the oily base weighed. The base was then dissolved in 30 cc. of alcohol and the exact molal quantity of ammonium chloride was dissolved in 8 cc. of water and added to the alcoholic solution of the base. This mixture was heated at 60–70° for three to four hours or until the mixture was neutral or slightly acid to nitrazine *p*H indicator paper. The reaction mixture was then concentrated and cooled. Acetone and ether were added until the mixture became turbid but no liquid layers separated. The reaction mixture was chilled in the refrigerator and fine needles of the amidine hydrochloride crystallized out.

The crystals were contaminated with unreacted ammonium chloride and they contained water of hydration. In order to remove the ammonium chloride and water of hydration, the product was converted to the free amidine base with a 10% solution of sodium hydroxide and extracted several times with chloroform. The chloroformic extracts were then combined and dried over anhydrous sodium sulfate. The chloroform solution was concentrated to a small volume and petroleum ether added to precipitate the free amidine base. The base was purified by recrystallization from benzene and petroleum ether. An ether solution of hydrogen chloride was added to an ether solution of the base which then yielded a crystalline precipitate of the amidine hydrochloride.

In *Method 2*, the ester of *p*-carbethoxyimidobenzoic acid hydrochloride was dissolved in 30 cc. of alcohol. Ammonium hydroxide was then added dropwise until a faint odor of ammonia persisted. The mixture was then heated at 60–70° for two hours, after which it was concentrated and cooled. After cooling, acetone and ether were added to t mixture until the solution became turbid. chilling, long needles of the amidine hydrochlor formed and were filtered off. The amidine hyd chlorides were purified in the same manner as o lined under Method 1.

Table IV gives the melting points of the free bas picrates and sulfates of the esters of *p*-carbo benzamidine:

p-Carboxybenzamidine Ester Carbonates.—: virtue of the strong basic character of the free an dines, they readily formed the carbonates in cr talline form when an aqueous solution of the an dine hydrochloride was neutralized with exc sodium bicarbonate solution. They were dried in vacuum desiccator over phosphorous pentoxide a recrystallized from absolute alcohol.

Terephthalic Acid Esters (VI).—The *p*-carbet oxyamidobenzoic acid ester hydrochlorides readi dissolved in water. On standing a turbidity d veloped which, in some cases, turned into a cryst line precipitate and, in others, settled out as an at 25°. The separated products were mixed or sy metrical terephthalic acid esters.

Terephthalamic Acid Esters (VII).—The cyanobenzoic acid esters were covered with 78

TABLE IV.—AMIDINES AND THEIR DERIVATIV

Ester	Amidine Base, M. p., ° C.	Amidine Picrate,[a] M. P., ° C.	Amidine Sulfate,[b] M. P., ° C.
Methyl	137–139	231–232	241–242 (decomp
Ethyl	110–112	213–214	252
Propyl	92–94	204–205	209–210
n-Butyl	63–64	192–193	199–200
n-Amyl	84–85	202–203	172–174
n-Hexyl	Oil (at 25)	195–196	210–211[c]

[a] Prepared in the usual manner from the free base.
[b] Prepared from the free base by neutralization.
[c] Prepared also from the ammonium sulfate reaction.

TABLE V.—AMIDINE CARBONATES

Ester	Melting Point, ° C.	Empirical Formula	Analysis, % C and H — Found	Calc.
Methyl	143–144	$C_{19}H_{22}O_7N_4$	C, 54.58 H, 5.65	C, 54.51 H, 5.30
Ethyl	108–109	$C_{21}H_{24}O_7N_4$	.....	.....
Propyl	102–103	$C_{23}H_{30}O_7N_4$	C, 57.77 H, 6.59	C, 58.21 H, 6.37
n-Butyl	86–88	$C_{25}H_{34}O_7N_4$	C, 59.49 H, 7.07	C, 59.74 H, 6.82
n-Amyl	91–92	$C_{27}H_{38}O_7N_4$	C, 60.73 H, 7.52	C, 61.11 H, 7.22
n-Hexyl	93–94	$C_{29}H_{42}O_7N_4$	.....	.....

TABLE VI.—TEREPHTHALIC ACID ESTERS

Ester	Melting Point, ° C.	Ester	Melting Point
Mono-ethyl	171 found 171 rep.	Propyl-ethyl	Oil
Methyl-ethyl[a]	35–36	*n*-Butyl-ethyl	Oil
Diethyl	44 found 44 rep.	*n*-Amyl-ethyl	Oil
		n-Hexyl-ethyl	Oil

[a] This compound was analyzed for $C_{11}H_{12}O_4$; calcd C. 63.45, H. 5.81; found C. 63.63, H. 5.97.

TABLE VII.—TEREPHTHALAMIC ACID AND ESTERS

Compound	Melting Point, °C.	Empirical Formula	—Analysis, % C and H— Found	Calc.
Terephthalamic Acid	Over 300 S at 250 rep. Over 360 found	$C_8H_7O_2N$	.....	.....
Methyl Ester	201 rep. 201 found	$C_9H_8O_2N$	.....	.....
Ethyl Ester[a]	171–172	$C_{10}H_{11}O_2N$	C, 61.98 H, 5.86	C, 62.16 H, 5.68
Propyl Ester[b]	131–132	$C_{11}H_{13}O_2N$	C, 63.72 H, 6.46	C, 63.75 H, 6.32
n-Butyl Ester[c]	131–133	$C_{12}H_{15}O_2N$	C, 64.93 H, 6.98	C, 64.84 H, 6.80
n-Amyl Ester	123–124	$C_{13}H_{17}O_2N$	C, 66.92 H, 7.53	C, 66.36 H, 7.32
n-Hexyl Ester	126–127	$C_{14}H_{19}O_2N$	C, 67.85 H, 7.88	C, 67.44 H, 7.68

[a] Formed also by hydrolysis of *p*-carbethoxyimidobenzamide hydrochloride.
[b, c] Formed also from the imido ester hydrochlorides by heating them under vacuum at their melting points.

sulfuric acid and allowed to stand for forty-eight hours. At the end of this time the mixture was poured on crushed ice and the amide crystallized out. The crude product was filtered off and washed with water and then purified by several recrystallizations from water. These compounds were obtained in yields of 60% to 65%.

p-Carbethoxyimidobenzoic Acid Hydrochloride.—Five grams of *p*-cyanobenzoic acid was suspended in 100 cc. of anhydrous ether and 25 cc. of absolute alcohol in a 250-cc. flask and chilled to 0°. Gaseous hydrogen chloride was passed in for one hour at a moderate rate. The reaction mixture was set aside in the refrigerator for several days at the end of which time the mixture was filtered and the residue washed with ether. The yield of product was 90% The melting point was over 300°, with decomposition and sublimation. In one attempt, only absolute alcohol was used as the solvent and the product isolated was the ethyl ester of *p*-carbethoxyimidobenzoic acid hydrochloride which formed through esterification of the free carboxyl group.

p-Carboxybenzamidine Hydrochloride. — One gram of *p*-carbethoxyimidobenzoic acid hydrochloride was dissolved in 20 cc. of a 10% solution of ammonia in absolute alcohol. After a few minutes the solution became turbid. After standing in sealed bottles for four days at room temperature, the precipitate was filtered off and the filtrate heated to remove the excess ammonia and to concentrate the liquid to small volume. The solution was then cooled and acidified with hydrochloric acid and the precipitate filtered off. Five-tenths of a gram of product was obtained by recrystallization from alcohol that melted at 283°.

Anal.: Calcd. for $C_8H_9O_2N_2Cl.H_2O$: C, 43.94; H, 5.07. Found: C, 44.37; H, 5.26.

p-Cyanobenzamide.—Five grams of *p*-cyanobenzoyl chloride was added in small portions to 20 cc. of ammonium hydroxide. The reaction was vigorous. Upon completion of the reaction, the mixture was cooled and filtered and washed with water. The insoluble residue, which was the amide when recrystallized from water, was obtained in the form of long slender needles. The yield was 2.9 Gm. of a product that melted at 224° which corresponded with that reported in the literature.

p-Carbethoxyimidobenzamide Hydrochloride.—Three grams of *p*-cyanobenzamide were dissolved in 100 cc. of absolute alcohol and the solution chilled to 0°. Gaseous hydrogen chloride was passed into the solution for thirty minutes. Twenty grams were absorbed. The solution was placed in the refrigerator for two days; then the crystals which formed were filtered off and washed with ether. The yield was 2.6 Gm. (71%). It melted above 300°.

Terephthalamamidine Hydrochloride.—*p*-Carbethoxyimidobenzamide hydrochloride (2.5 Gm.) was dissolved in alcohol and warmed while ammonium hydroxide was added in the manner described under Method 2. The mixture was heated for several hours at 60–70° at the end of which time the solution was concentrated. Ether was added and the amidine hydrochloride precipitated out. One gram of product that melted at 284–285° was obtained by recrystallization from alcohol.

Anal.: Calcd. for $C_8H_{10}ON_3Cl$: C, 48.12; H, 5.09. Found: C, 47.66; H, 4.96.

SUMMARY

Six new amidines, containing ester groupings in their structure, were prepared, together with some of their derivatives.

Five new esters of *p*-cyanobenzoic acid were prepared in good yields.

Five new esters of terephthalamic acid were prepared from the corresponding nitriles and the imido ester hydrochlorides.

Mixed esters of terephthalic acid were prepared by hydrolysis of the corresponding imido ester hydrochlorides. The mono-ethyl, methyl-ethyl, and diethyl are solids, the propyl through hexyl-ethyl esters were obtained as oils.

The method of Pinner as modified by Knorr and Barber for the preparation of unsubstituted amidine salts has been applied to nitriles containing ester groups in converting them to amidines in high percentage yields. It was found that the water used to dissolve the ammonium chloride in this method was unnecessary.

An improved method for the preparation of amidines from the imido ester hydrochlorides was devised. The yields are comparable to or greater than the previous method. The conversion of the imido ester hydrochloride to the base and subsequent extraction with ether preparatory to reaction with ammonium chloride in a hydro-alcoholic solution is entirely eliminated by the direct addition of ammonium hydroxide to the imido ester hydrochloride in alcohol.

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The Spectrophotometric Determination of the Dissociation Constants of Theophylline, Theobromine, and Caffeine*

By ARTHUR TURNER, Jr.,† and ARTHUR OSOL‡

The results of a study of the ultraviolet spectrophotometric absorption characteristics of aqueous solutions of theophylline, theobromine, and caffeine at different *p*H values are reported. From these data the acidic dissociation constants of theophylline and theobromine, expressed as pK_a values, were found to be 8.6 and 10, respectively. The basic dissociation constants of each of the three substances correspond to a pK_b value of not less than 13 in any case.

THEOPHYLLINE (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), and caffeine (1,3,7-trimethylxanthine) all behave as weak bases. Theophylline and theobromine, each having an —NH— group in its molecule, can also function as acids; caffeine, not having such a group, cannot react as an acid. The possible mechanisms of acid behavior of theophylline and theobromine have been discussed by Ogston (1), who also measured their acidic dissociation constants potentiometrically.

A study of the ultraviolet spectrophotometric absorption characteristics of the three xanthine derivatives at different *p*H values appeared to be of interest, especially from the standpoint of the possibility of determining the acidic and basic dissociation constants of theophylline and theobromine, and the basic dissociation constant of caffeine. If the absorption characteristics of the compounds produced by the addition of alkali or acid are different from those of the "neutral" forms it is possible to calculate the dissociation constants from a knowledge of the variation of the optical density of a solution of the substituted xanthine with the *p*H of the solution.

EXPERIMENTAL

Theophylline, theobromine, and caffeine were recrystallized three times from hot distilled water and dried at 100°. All solutions of these substances contained 20 mg. per liter and were prepared with distilled water. Adjustment of reaction in the range of *p*H approximately 7 to 10 was effected by inclusion of Clark and Lubs buffer components; more strongly acid or alkaline solutions were adjusted by adding hydrochloric acid or carbonate-free sodium hydroxide solution. In every case compensation for absorption by acidifying or alkalinizing agents was made by the use of appropriate blanks.

All spectrophotometric measurements were made using a Model DUV Beckman Quartz Spectrophotometer. Fused silica cells, containing 1.000 cm. thickness of solution, were employed and all measurements were made at a slit width of 0.80 mm. Measurements for the determination of *p*K values were performed at wave length 240 mμ, where a pronounced vertical shift of the absorption curves of the xanthine compounds was observed. *p*H measurements were made before and after spectrophotometric measurement with a Leeds and Northrup *p*H meter, employing glass and saturated KCl-calomel electrodes. The temperature at which all observations were made was 25°.

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† Based upon the thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Chemistry, by Arthur Turner, Jr. (present address, Eastern Regional Research Laboratory, Philadelphia, Pa.).
‡ Professor of Chemistry and Director of the Department of Chemistry, Philadelphia College of Pharmacy and Science.

RESULTS AND DISCUSSION

Figure 1 shows the spectral transmission curves for aqueous solutions of theophylline, theobromine, and caffeine; the recorded *p*H is that of the solution without special adjustment.

Figure 2 shows the effect of variation of *p*H on the spectral transmission curve of theophylline. It is apparent that a pronounced vertical shift, notably at wave length 240 mμ, occurs. This is due to the different absorption characteristics of the different forms of theophylline obtained by the addition of acid or alkali. Similar curves were obtained for theobromine and caffeine, except that with the latter substance no significant displacement was observed on adding alkali, an observation expected from the

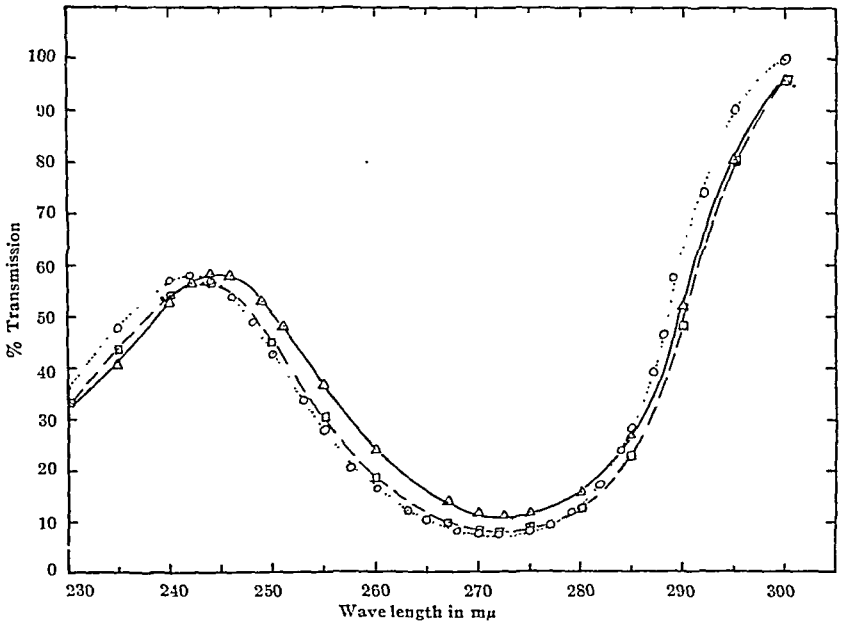

Fig. 1.—Spectral transmission curves of Theophylline, Theobromine, and Caffeine. ⊙, Theophylline *p*H 6.4; ⊡, Theobromine *p*H 5.7; △, Caffeine *p*H 5.6.

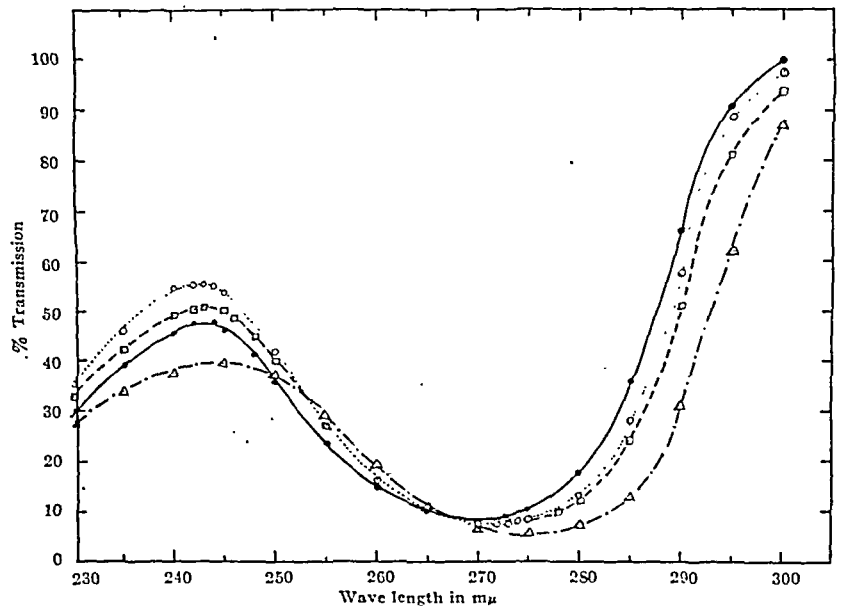

Fig. 2.—Change in spectral transmission curves of Theophylline at various pH values. ●, pH 1.0; ⊙, 6.4; ⊡, 8.6; △, 12.5.

fact that caffeine cannot react as an acid. When conversion to a single entity is complete the absorption curve undergoes no further change upon addition of acid or alkali, as the case may be.

If the optical density of each of a series of solutions of constant concentration but different pH is determined at a wave length where a pronounced spectral shift occurs (in this case 240 mμ), the curves shown in Fig. 3 may be derived. Analysis of these curves in the region representing alkalinized solutions permits quantitative evaluation of the acidic dissociation constants of theophylline and theobromine; the mid-point of the S-shaped portion of the curves for these substances in the alkaline region is the pH at which the form produced by alkali and the "neutral" form are present in equimolar quantities selected graphically represents the optical density of equimolar quantities of two forms of theophylline and theobromine, respectively, and therefore also the pK_a of each of these xanthines may also be checked by the equation of Beadle and Zscheile (2) for the resolution of two-component systems from spectrophotometric data. Their equation states:

$$\%\ \text{Alkali-produced form of the xanthine} = \frac{(D/C) - k_1}{k_2 - k_1} \times 100$$

where D = optical density at the pH considered to be equal to pK_a, C = total concentration of both forms of the xanthine in Gm. per liter, k_1 = specific extinction coefficient for the "neutral" form of the

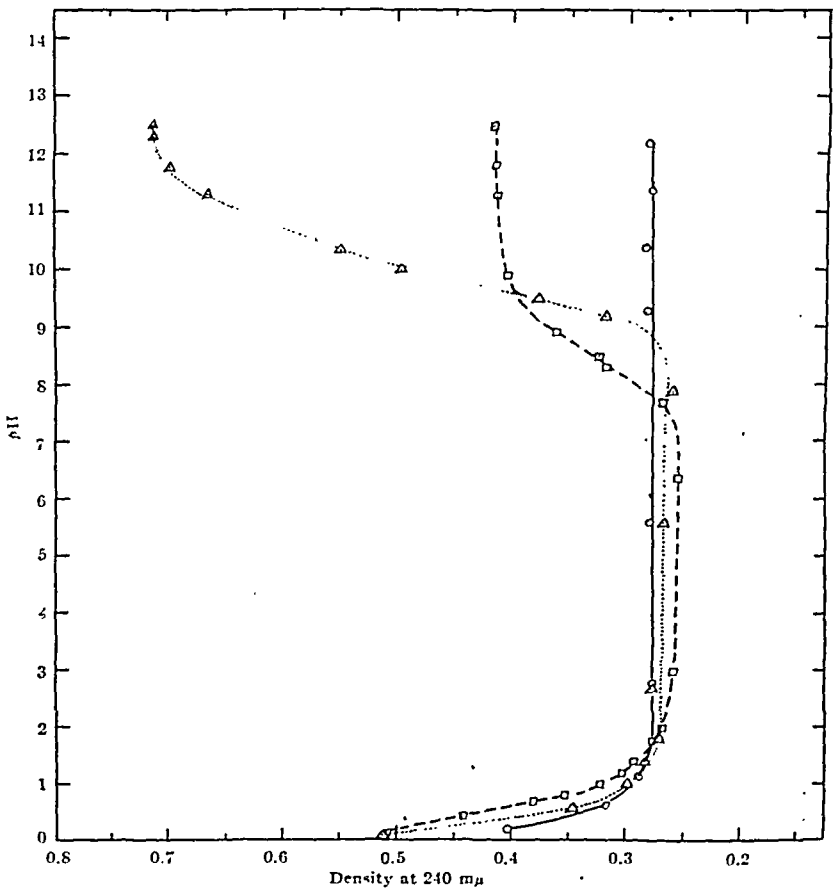

Fig. 3.—Curves showing variation in density with pH. Concentration 0.020 Gm. per liter. △, Theobromine; ⊡, Theophylline; ⊙, Caffeine.

This pH is numerically equal to pK_a, from the fundamental relationship:

$$pK_a = pH + \log \frac{[\text{form produced by alkali}]}{[\text{"neutral" form}]}$$

The pK_a values for theophylline and theobromine, graphically obtained, are 8.6 and 10.0, respectively; these are in good agreement with the values of 8.6 and 9.9, respectively, obtained from potentiometric measurements by Ogston (1). That the mid-point

xanthine, and k_2 = specific extinction coefficient for the alkali-produced form of the xanthine. The values for theobromine, namely, D = 0.505, k_1 = 14.1, k_2 = 36.4, and C = 0.02, lead to 50.2% of theobromine in alkali-produced form at the mid-point of the curve in Fig. 3. For theophylline the values D = 0.350, k_1 = 13.5, k_2 = 21.5, and C = 0.02 lead to a proportion of 50.0% of alkali-produced form of theophylline at the mid-point of its curve.

Since the straight-line portion of the curve for caffeine (in Fig. 3) is not significantly altered in the

alkaline region it may be concluded also from this experiment that the substance does not react as an acid.

Inspection of the curves in Fig. 3 in the region of acidified solutions shows that all three xanthine derivatives undergo reaction with acids beginning at about pH 2. Although measurements were made on solutions having an apparent pH as low as 0.2, the optical density did not attain a constant value, which would have been indicative of complete conversion to a single entity. The most that can be said is that the basic dissociation constants for theophylline, theobromine, and caffeine, respectively, correspond to a pK_b of not less than 13.

SUMMARY

1. The ultraviolet absorption spectra of aqueous solutions of theophylline, theobromine, and caffeine have been investigated, including also a study of the effect of variation of pH on the spectra.

2. From these data the acidic dissociation constants of theophylline and theobromine, expressed as pK_a values, have been found to be 8.6 and 10.0, respectively; caffeine has been found also by this method not to function as an acid. The basic dissociation constants of the three substances correspond to a pK_b value of not less than 13 in any case.

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Conductometric Studies of Certain Salts of Penicillin*

By FRANK M. GOYAN†

A one-tube balanced amplifier and rectifier for visually detecting the minimum signal of a typical Jones and Josephs conductance bridge is described. This circuit increases the maximum sensitivity of the bridge, but is under complete control of the operator who may select any degree of sensitivity desired. The apparatus described is suitable for work of high precision or for routine conductometric titrations. A technique is developed for conductometric investigation of solutions of salts of benzyl penicillin; the results show graphically the degree of ionization of the different salts studied.

THE WORK of Woodbury and Rosenblum (1) on the conductance of solutions of sodium benzylpenicillinate established the fact that this salt behaves as a completely dissociated electrolyte of the 1:1 valence type at 30° up to a concentration of about 0.05 *M*. Kumler and Alpen (2) confirmed these findings by extending the investigation to include other salts, namely potassium and aminoacridine benzylpenicillinates. Additional work in this laboratory has substantiated further the conclusion that benzylpenicillinate ion in water solution is a typical, large, univalent, negative ion.

While it is entirely possible that certain combinations of ions with the benzylpenicillinate ion in solution may offer decided advantages or disadvantages, in terms of biological activity or otherwise, it is immaterial what pure salts are dissolved to provide the desired concentrations of the various ions under consideration. For example, a solution of potassium benzylpenicillinate and sodium chloride may be identically the same as a solution of sodium benzylpenicillinate and potassium chloride. It is assumed, for the purpose of this argument, that traces of impurities associated with the different solid salts may be eliminated from consideration; however, this assumption is not always justified in practice.

The difficulties involved in preparing any given salt of penicillin suggest the desirability of extending the study of different salts to mixed electrolytes which can be prepared from available pure salt of penicillin by the addition of a second strong electrolyte. For example, the behavior of sodium benzylpenicillinate may be inferred from a study of solutions of potassium benzylpenicillinate and sodium chloride by well-known methods of conductometric titration (3, 4). These titrations involve making many

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conductivity determinations, one after each addition of a small uniform sample of a fairly concentrated solution of one of the electrolytes of the mixture. However, before proceeding with this general method, it was necessary to overcome technical difficulties which evidently have hindered a more general application of conductance measurements to the solution of problems involving substances of pharmaceutical interest. Studies of this nature often require a precision of measurement somewhat greater than is easily obtained with one of the instruments designed exclusively for conductometric titrations (5).

It is extremely arduous to undertake many routine measurements involving the use of a bridge method requiring detection of the point of balance by estimating a condition of minimum sound in earphones. As a matter of fact, the author is convinced from his own personal experience that there is a very real element of danger to the operator in using audio methods because of the temptation to increase amplification well above room noise level at the time the point of bridge-balance is reached. As a result of this excessive amplification, there is a tendency to allow dangerously loud vibrations to reach the ear when the bridge is accidentally thrown out of balance. To be sure, this danger may be overcome to a large degree by proper amplifier design; however, it is desirable to make use of visual methods of detection if possible.

The design of a balance detector of the visual type to be used with existing facilities, consisting of a Jones and Josephs type bridge with electronic oscillator and amplifier originally intended for audio detection, was so successful that it became clear that routine measurements of electrical conductance with any desired degree of precision were entirely practicable. In fact, the circuit developed to meet this need, presented schematically in Figure 1, makes it possible to increase the precision of the existing bridge by a factor of ten, when desired, without sacrificing the ease or speed of attaining approximate balance within predetermined limits. There is, of course, the usual psychological problem arising from the fact that an interested operator is seldom content with less than the maximum precision of the instrument. However, this problem can be overcome by adjusting the work to a time schedule.

EXPERIMENTAL

Standard Electrical Equipment.—The conductivity bridge used in this work was constructed from component parts purchased over a period of years

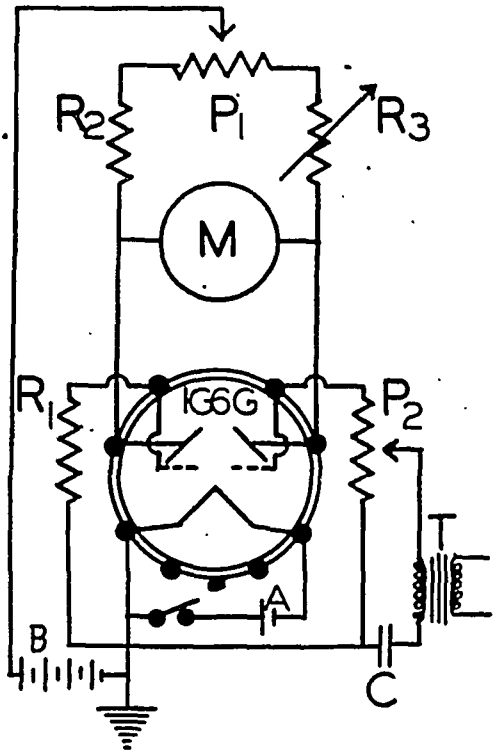

Fig. 1.—Visual Balance Indicator Circuit Diagram. *A*, 1.5-v. "A" battery; *B*, 90-v. "B" battery; *C*, 0.005 m. f. d. or larger; *M*, microammeter (0–100 microamp. with six-inch dial used but not required); P_1, 500 ohms; P_2, 1 megohm; R_1, 1 megohm; R_2, 7000 ohms; R_3 0–10,000 ohms.

Up to the time of the introduction of the visual balance indicator, the assembly of a General Radio Ratio Arm Box (type 610-A), a six-dial Leeds and Northrup 10,000-ohm decade resistance box and vacuum tube oscillator (Cat. No. 9842), together with necessary resistors and condensers, represented a typical Jones and Josephs bridge (6) with a Wagner earthing device. A vacuum tube amplifier of conventional design was coupled to the bridge by means of a suitable impedance matching transformer. This circuit has remained unchanged with the introduction of the visual balance indicator.

Visual Balance Indicator.—The new visual balance indicator was designed to replace the phones in the existing circuit. Instead, it replaces one of a pair of phones; the other phone remains in series with the primary of the transformer T (Fig. 1). This arrangement has proved to be desirable in teaching; the single phone is never placed near the ear, but it is easily heard when the bridge is far off balance. Because of the fact that the amplifier and oscillator are powered from the 115-v. a.-c. source, it was decided to power the visual balance indicator with batteries to avoid possible difficulties with the ground connections. The filament of the 1G6G tube (Fig. 1) operates for many hours from one 1.5-v. No. 6 dry cell. Two 45-v. radio B batteries in series supply the plate voltage. The size of the B batteries is not critical because the total plate current drain is less than 3 ma. The meter M

(Fig. 1) is a six-inch dial Triplett microammeter (range 0–100 microamperes). This meter is very convenient because it is easy to see from any position near the instrument, but any galvanometer of equal or greater sensitivity will serve the purpose. The potentiometers, P_1 and P_2 are the principal controls: R_3 is adjusted so seldom that it need not be placed on the front panel. The transformer T is an old audio frequency transformer with a 3:1 ratio introduced to provide insulation from unwanted ground connections of the amplifier circuit rather than for critical impedance matching. Condenser C was introduced with a similar purpose in mind, but the circuit seems to work well when this condenser is shorted.

Shielding.—Various components of the entire bridge seem to be critical with respect to position. For example, it is necessary to place the input transformer a foot or more away from the amplifier because of the power transformer built into the amplifier cabinet. All of the metal cases of the various components of the bridge are grounded, and some of the lead wires are shielded. Shielding is less critical with the visual balance indicator because of the fact that it is possible to observe the point of minimum deflection against any steady background noise. However, reduction of noise level is important when maximum sensitivity is demanded. Considerable loss of efficiency is noted when certain fluorescent lamps are operated on the same table. A tuned amplifier or band-pass filter is indicated but has not as yet been added to the circuit.

Thermostats.—An oil thermostat is available for special work; however, for reasons of convenience, a water thermostat was used for this work. The thermostat is maintained at 25° within 0.01° by means of a mercury regulator and relay. On warm days an air blower playing over the surface of the water serves to prevent loss of control. Work at or near 0° is done in a vacuum jar filled with chopped ice and covered with a fitted plastic box also filled with ice.

Titration Cell.—A Leeds and Northrup Henry type conductivity cell (7) with lightly platinized electrodes is used as a titration vessel. A cell constant of 0.208 cm.$^{-1}$ was determined for the cell when filled with 0.001 M KCl to a predetermined mark representing a volume of 26.7 cc. Stirring after each addition of electrolyte is accomplished either by bubbling washed nitrogen from a glass capillary which must be removed before balancing the bridge, or by means of a glass propeller-type stirrer rotating near the surface of the liquid. The glass stirrer operates well above the electrodes and need not be removed to balance the bridge.

Operation of the Bridge.—Resistance measurements of the filled cell are made by conventional methods involving selection of suitable ratio-arm settings followed by adjustment of the Wagner earthing device, and finally by adjustment of the decade resistance box to position of bridge balance. The amplifier is permanently set to a high stable value; control of sensitivity is achieved by means of the oscillator volume control and the potentiometer P_2 (Fig. 1). When the visual balance indicator is first put into service R_3 is adjusted so as to cause the meter to read between zero and midscale, while P_1 is near the center of its range and P_2 is at its ground or insensitive position. The meter is then set to zero with P_1, and finally P_2 is adjusted to give a high meter reading. As the balance point is reached the meter reading approaches zero. When additional sensitivity is required, P_2 is further adjusted toward higher sensitivity, and more power is supplied from the oscillator until the meter again reads a high value. Further adjustment of the bridge will cause the meter reading again to approach zero. The lowest meter reading indicates the point of best bridge balance. For rapid conductometric titrations it is desirable to arrange the sensitivity of the visual balance indicator so that the only sensitivity control needed is the volume control of the oscillator which was operated at 1000 cycles per second.

Titration Techniques.—All electrolytes studied were made up in distilled water to a concentration of 0.1 N. The titration vessel was filled to the predetermined mark with redistilled water which was checked to avoid the possibility of contamination at the start of each titration. The first electrolyte was added to the water from a micro-pipette designed to deliver 0.1 ml. After each addition, the bridge was balanced and the resistance recorded. After the addition of 0.4 ml. of 0.1 N solution of the first electrolyte, the second electrolyte was added using the same pipette, and resistance measurements were made after each addition until 0.7 or 0.8 ml. had been added. Care was taken to maintain uniformity of volume and of concentration, but little attention was paid to absolute accuracy. The potassium benzylpenicillinate used was of good quality but not the best available (1500 units per milligram), and the other salts were of C. P. or reagent quality. The mercuric nitrate was dissolved in 0.02 N nitric acid to prevent precipitation of basic salts. The solutions were made with some care but only the potassium chloride was dried before weighing. The concentrations of the other solutions may be less than the nominal value, a matter of no importance in the present study.

RESULTS

The results of several conductometric titrations are presented graphically in Fig. 2. In all cases the reciprocals of the measured resistances in ohms were read from an ordinary ten-inch slide rule and plotted against the volume of solution added. The curves shown are sufficient to illustrate the method. It will be noted that only the hydrochloric acid curve and the mercuric nitrate curve show marked curvature. The formation of a moderately weak acid is to be expected and is accounted for by the observed shape of the curve. The titration with mercuric nitrate caused the formation of a white precipitate that failed to redissolve upon the addition of more reagent or of nitric acid. The nature of this precipitate cannot be deduced with certainty from the curve, but it is interesting to note that the straight line portion begins at the point corresponding to chemically equivalent amounts of mercury and pencillin. This would suggest a salt of the type, HgP_2, where P represents the univalent benzylpenicillinate radical.

The fact that the other curves approach straight lines in those portions representing the addition of the second electrolyte (0.4 cc. except for the HCl

curve which begins at 0.5 cc.) indicates that the corresponding salts are normal strong electrolytes.

DISCUSSION

It can be shown from the fundamental definitions of equivalent conductance and specific conductance (7) that the reciprocals of resistance values (obtained with the same conductivity cell and the same electrolyte in solution) are proportional to concentration of the electrolyte if its equivalent conductance remains constant. Actually there are two compensating errors that make the points shown in Fig. 2 fall on straight lines as nicely as they appear to do. The equivalent conductance values are not strictly constant, and volume of added reagent is slightly different from a linear function of concentration because, with each addition of reagent, there is a corresponding dilution of the entire solution due to

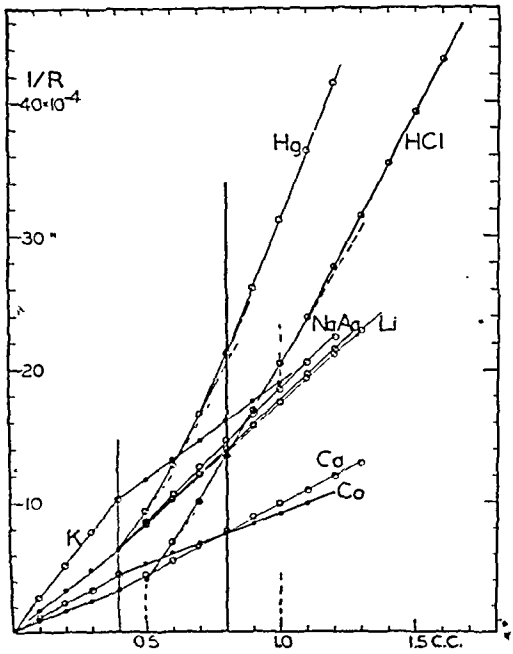

Fig. 2.—Results of Conductometric Titrations. Small solid points represent addition of fresh 0.1 *N* solution of potassium benzylpenicillinate. Larger circles represent 0.1 *N* solutions of different salts as follows: At 0° C. Ca = $CaCl_2$, Co = $CoCl_2$, HCl = HCl. At 25° C. K = KCl, Na = NaCl, Ag = $AgNO_3$, Li = LiCl, Hg = Hg $(NO_3)_2$ in 0.02 *N* HNO_3. (Initial volume of water = 26.7cc.)

the slight increase in volume, superimposed, of course, upon the larger increase in concentration.

Although it is not difficult to compensate for the dilution effect in plotting, thereby permitting a calculation of equivalent conductance within the limits of volumetric accuracy at any concentration, this is not done because it is felt that the principal value in this method lies in its simplicity. The slope any line is taken as an empirical measure of t equivalent conductance of the electrolyte bei added or being formed by chemical interaction. the case of titration with hydrochloric acid, if t weak acid formed were a very weak electrolyte, t curve would have the slope shown for potassiu chloride. If no weak acid were formed, the slo of the HCl line between the 0.5-cc. point and t 1.0-cc. point would be the same as that of hydr chloric acid shown beyond, and there would be a break in the curve. Although later work m demonstrate the value of more exact analytic treatment of the data, the principal interest at tl present time is to discover abnormalities in solutio that may suggest need for further investigation in broader sense than might be attempted by refin ment of conductance measurements.

SUMMARY

The study of certain salts of benzylpenicillinat is undertaken by a method of conductometri titration. Results are presented graphicall showing that lithium, sodium, potassium, silver calcium, and cobalt salts of benzylpenicillinat show no marked deviation from the beltavio of strong electrolytes in dilute aqueous solutions. The formation of a weak acid is verified by the curves presented, and the formation of a white precipitate of some mercury salt is reported and correlated with the corresponding curve.

The potential serious danger to the hearing of operators of sensitive bridges depending upon detection of balance by estimating minimum sound in phones is mentioned as one reason for the development of a visual balance detector that may be used to improve the sensitivity of a typical Jones and Josephs bridge. Circuit details are given and directions are included for using the new visual method with any degree of precision desired. The visual method of detection of balance may be used for rapid approximate measurements because the point of balance can be detected over background noise.

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Routine "Safety" Tests on Drug Products and the Organization and Maintenance of a Requisite Mouse Colony*

By JOHN H. BREWER†

ALTHOUGH required by the National Institute of Health for most of the biological products manufactured under their license, many injectable preparations on the American market are not subjected to routine safety testing in animals. On many of these products the manufacturer depends entirely on routine analytical and sterility tests, and the final pharmaceutical preparation is not actually tested for toxicity before being released for sale.

In our manufacturing processes we have believed that a routine safety test would give us a feeling of security and also be a check on our manufacturing, sterility, and control laboratories. We have determined for each ampul preparation manufactured by us, based on the human dose, the human equivalent dose for white mice. We record in our testing the reaction in mice to the human equivalent dose. We also determine the maximum dose which can be given to 20-Gm. white mice without producing death. From this is calculated and recorded the "Safety Factor" which represents the magnitude of the dose as compared to the equivalent human dose. The products are injected either intravenously by tail vein injection or intraperitoneally in the case of oil preparations. Ten ampuls from each lot manufactured are submitted to these tests and the animals are observed for seven days. This, along with other tests, is released on the same day as our routine sterility test and thereby does not hold up the production of any ampuled product, unless unwarranted toxicity is found.

TESTS APPLIED

For some of our products, for instance BAL in Peanut Oil, we can give only 9.75 times the equivalent human dose without reaction. In the case of other preparations, however, the entire human dose may be given, which in some instances is 3500 times the human equivalent dose in mice, without any sign of reaction. For instance, in the case of phenolsulfonphthalein, the human equivalent dose would be 1 cc. of a 1:3500 dilution of the phenolsulfonphthalein. This produces no reaction in mice. In our routine animal safety tests, however we give 1 cc. of the full strength material. This is 3500 times the human equivalent dose and produces no reaction in mice.

The route of injection is by tail vein and since this is the actual human dose, the lethal dose is not determined. In the case of many preparations which produce death, however, we do determine such minimal lethal dose (or LD_{50}) on each lot, or rather determine that this *M. L. D.* for any particular lot is not greater than the average for this particular product. Bromsulphalein is a good example of this type preparation. The average human dose ampul size is 3 cc. The equivalent human dose in mice is 1 cc. of a 1:1150 dilution. This dosage produces no reaction in mice. In our routine tests, however, we administer 0.1 cc. of the full strength material intravenously and this is 115 times the equivalent human dose based on a 2 mg./Kg. dosage. It would be 46 times the equivalent human dose if 5 mg./Kg. were used. This material is given slowly and very carefully to produce no necrosis and no reaction in the animal. However, if injected rapidly so that some of the material goes intrastitially, it may cause necrosis or even death of the animal. Lethal dose of this material for 20-Gm. white mice is 0.18 cc. of the full strength material. A typical chart of these products is shown in Table I.

This type of testing simulates the safety test required by the National Institute of Health for products made under its license and gives a feeling of security concerning our preparations which we would not otherwise have, since it is entirely possible that a product may pass the routine sterility tests and also chemical analysis, and still cause unfavorable reactions when injected, particularly in the case of some of the products whose therapeutic index is very low. In addition, such tests serve to eliminate any lots which might conceivably produce reactions from other causes not connected with the drug itself. These tests can be conducted at the same time the other routine tests are done and do not hold up our production in any way.

Mouse Colony.—The mouse colony described is small but practical and its primary purpose is to supply a sufficient number of mice to be used for routine safety and toxicity tests. Proper equipment is possibly the most important factor in a successful mouse colony. Stainless steel is the ideal material for all units. Aluminum may also be used advantageously. However, if aluminum is used, one must make all openings small so that the mouse cannot get his teeth through and gnaw his

* Received February 2, 1949.
† Director of Biological Research, Hynson, Westcott &
nt for suggestsign, and Mr. to the operation of the colony.
The stainless steel equipment was manufactured by the Baltimore Biological Laboratory.

TABLE I.—ANIMAL CONTROL TESTS OF STERILE PRODUCTS

Product	Human Dose (According to Ampul Size)	Animal Dose (Mice) (Equivalent of Human Dose)	Reaction in Mice to Human Equivalent	Dose Administered in Routine Test	Routine Test Is Number of Times of Human Dose	Reaction to Routine Dose	Route of Injection	Lethal Dose
BAL in Peanut Oil	4.5 cc.	1 cc. of 1:780 dilution	No reaction	0.25 cc. of 1:20 dilution	9.75 times	No reaction on injection	Intraperitoneally	0.5 cc. of 1:20 dilution
Phenolsulfonphthalein	1 cc.	1 cc. of 1:3500 dilution	No reaction	1 cc. full strength	3500 times	No reaction	Tail vein	Not determined
Bromsulphalein	3 cc. (2-mg. dose)	1 cc. of 1:1150 dilution	No reaction	0.1 cc. full strength	115 times	Give slowly; may cause necrosis in tail	Tail vein	0.18 cc. full strength

way out. The dimensions and figures given below have been obtained from our colony and could be changed to suit other conditions without altering the utility.

The breeder box (Fig. 1, *A*), as used in this colony, measures nine and one-half inches wide, twenty inches long, and four and one-half inches deep. This is fitted with a perforated top (Fig. 2, *A* and *B*), which is designed to hold a wire mesh feeder that will extend through to the underside, and a rack to hold water bottles, the ends of which will also extend through to the underside. These units can be fed and watered from the top without removal of the lid, thereby greatly reducing the time required for the daily feeding and watering of the colony.

A practical stock box (Fig. 1, *B*), which is used in conjunction with the breeding colony, measures seven inches wide, ten inches long, and six and one-half inches deep; it is fitted with a wire mesh top which contains the wire mesh feeder and the water bottle rack (Fig. 2, *C* and *D*). This box is not only used to house the stock mice weaned from the breeder units but also for the mice during the tests.

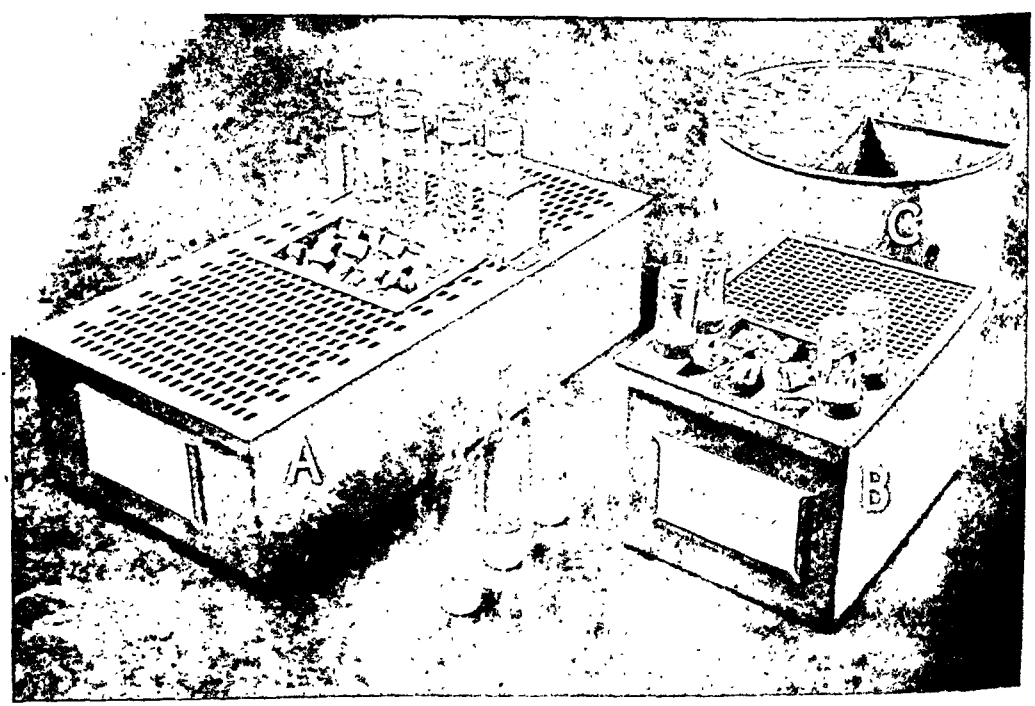


Fig. 1.—Breeder box, stock box and sterilizer pan.

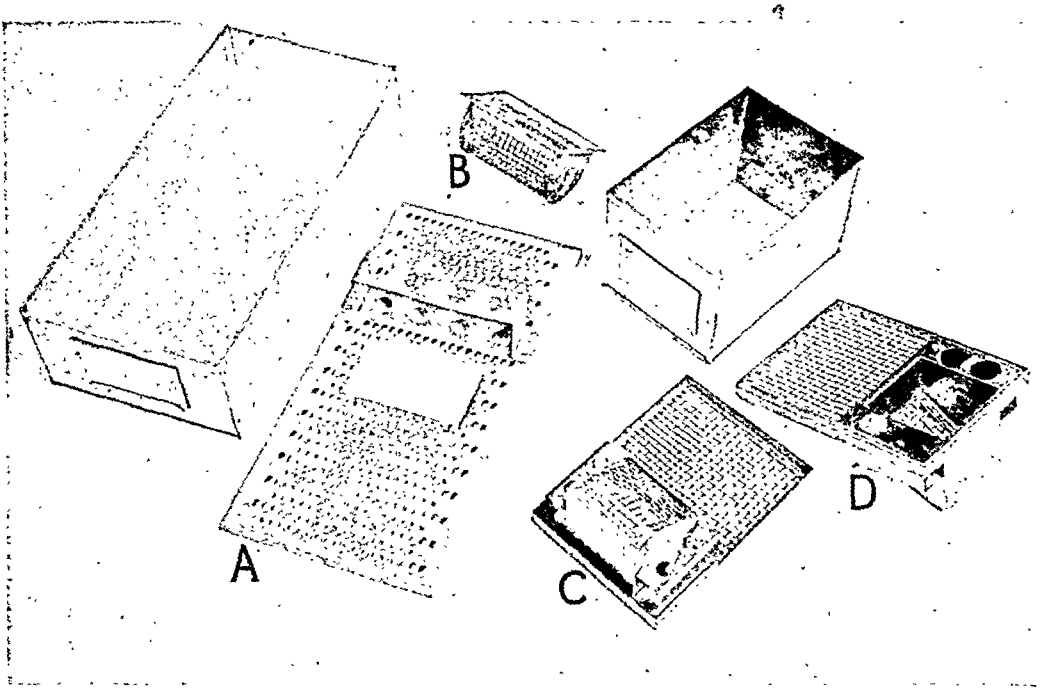


Fig. 2.—Expanded view of breeder box and stock box.

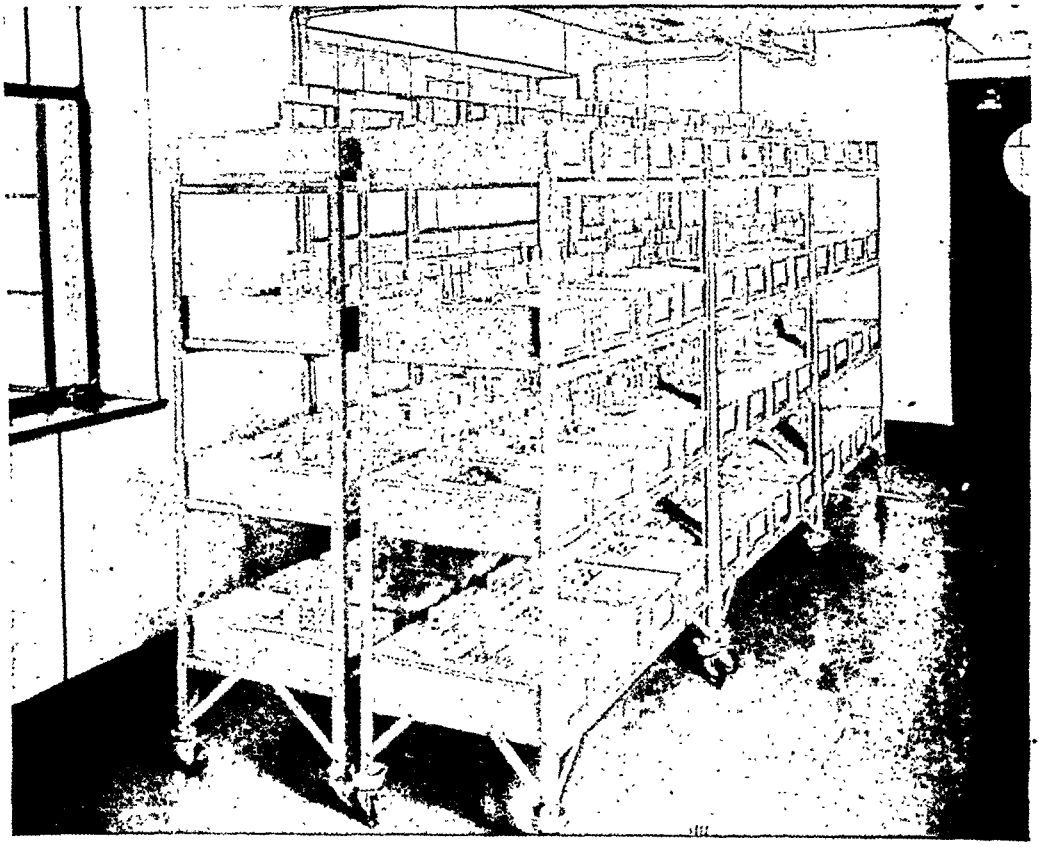


Fig. 3.—Breeder units.

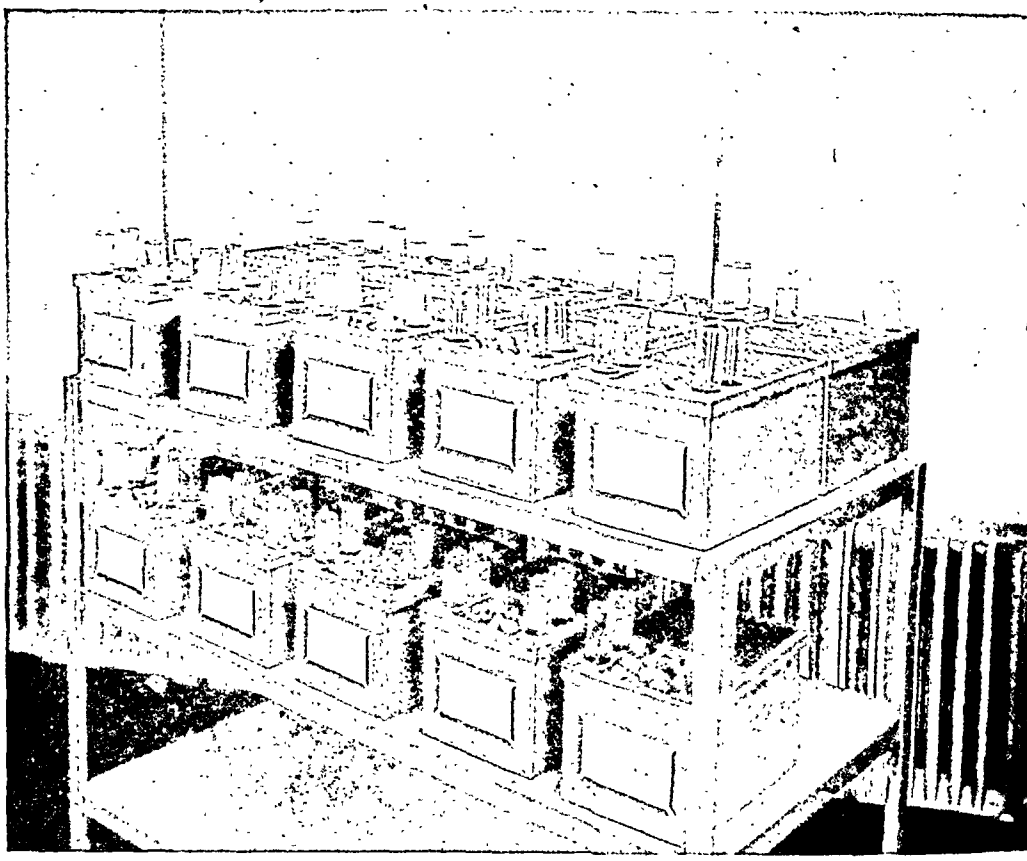


Fig. 4.—Stock boxes and mice under test.

The water bottle mentioned above is a standard (Kimble) fifty milliliter ampul with the mouth flamed in such a manner as to produce a small opening (3.5 mm.). When inverted into the rack, clean water is readily available to the animals merely by licking the lip of the ampul. Four of the ampuls are supplied to each breeder unit. The water can be automatically filled into the ampuls and sterilized in one operation with the use of suitable pans (Fig. 1, *C*) and an autoclave. The pans, containing the empty, inverted ampuls are partially filled with water and placed into the autoclave. The steam pressure is raised to twenty pounds and held there for twenty minutes. The amount of water to be filled into the pans is governed by holes in the pan about two inches from the top. Upon cooling, a partial vacuum is formed in the ampuls and this space is in turn filled with the water in the bottom of the pan as the ampuls cool.

Based on individual experiments, it has been estimated that the average mouse will drink approximately four milliliters of water and eat four grams of food daily (Purina Lab. chow).

The breeder units are kept on a four-shelved dolly (Fig. 3); each shelf is large enough to accommodate four breeder units. This comprises a sixteen unit "group." Six of such "groups" are in operation in this model colony. The stock boxes and mice under test are kept on similar dollies (Fig. 4).

Both the stock and breeder boxes are furnished with about one-half inch of fine sawdust for bedding. All boxes are washed in a warm chlorine solution twice a week and fresh sawdust supplied.

The time requirements for the care and maintenance of this colony are as follows: changing stock and breeder boxes, washing boxes, and replacing sawdust—two persons two and one-half hours twice a week; daily feeding and watering, one person approximately two hours; weaning of young, one person approximately one-half hour per group once a month.

Now that the proper equipment has been procured and arranged in a suitable manner, it is a relatively simple matter to establish the breeding colony. Seven females and one male comprise the family and a family is placed in each of the breeder units previously mentioned. The young from these units are weaned once a month, a good average yield being ten. However, this number may vary from five to twenty-five or more, depending on the age and condition of the breeders and the correlation of the gestation cycles.

The colony mentioned above consists of six groups in three pairs, a total of ninety-six breeder units. Each pair of groups, or thirty-two units, is weaned once a month. When at the peak of production, this colony will produce approximately two hundred usable mice per week. If the production of any one unit falls off considerably, it is

advisable to replace the entire unit with new mice because the malfunction of this one unit will naturally bring down the average of the whole group. The highest productive age span for the average mouse is from two to ten months; after ten months the production drops off and it is well to replace the unit with young mice.

A very important factor is the temperature of the room in which the colony is housed. This temperature must be fairly constant, ranging from 75° to 80° F. If this temperature is not maintained, serious damage or death may result to the breeding animals and the average output of young is considerably lessened.

Although the stainless steel equipment shown in the accompanying photographs is relatively expensive when compared with galvanized boxes, it will outlast several sets of galvanized cages and withstands sterilization and chemical agents without corrosion or discoloring.

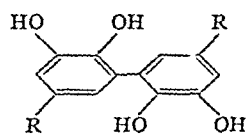
The Synthesis of Some Effective Antioxidants and Antiseptics*

By AKIRA ASANO† and OLE GISVOLD‡,§

Certain guaiacol derivatives can be readily coupled to yield the corresponding dehydrobicompounds which in turn can be readily converted to the corresponding dehydrobicatechols. Therefore, the following 2,2′,3,3′-tetrahydroxy biphenyls were synthesized, i.e., 5,5′-dimethyl (I); 5,5′-di-*n*-propyl (II); 5,5′-di-*n*-amyl (III); and the methyl (IV), ethyl (V), and normal propyl (VI) esters of the 5,5′-dicarboxy compound. Compounds I, II, and VI exhibited very powerful bacteriostatic activity that was inhibited by blood serum. The alkyl derivatives I, II, and III exhibited marked antioxidant activity in lard when tested by the Swift rapid rancidity method. The esters IV, V, and VI gave poor protection.

It is known that eugenol easily undergoes oxidative coupling with ferric chloride to yield dehydrodieugenol (2,2′-dihydroxy-3,3′-dimethoxy-5,5′-diallyl biphenyl). This type of coupling with eugenol was first reported in 1908, by Cousin and Herissey (1), who investigated the dehydrogenation of eugenol with an oxidizing enzyme and with ferric chloride. Certain other phenolic compounds also undergo oxidative coupling with various oxidizing agents.

Nordihydroguaiaretic acid (2, 3) and alpha, omega-bis(3,4-dihydroxyphenyl) alkanes (4) have been shown to possess marked antiseptic and antioxidant properties. This suggested the synthesis of 4,4′-disubstituted 2,2′,3,3′-tetrahydroxy biphenyls as possible useful antiseptics and antioxidants. Since 4-substituted guaiacols, such as eugenol and vanillin, couple in the unoccupied orthoposition to the hydroxyl group to form the corresponding bis compounds, the preparation of substituted dehydrobicatechols was undertaken by first coupling certain 4-substituted guaiacols. The following substituted dehydrobicatechols were prepared:



where R = CH_3, $CH_2CH_2CH_3$, $CH_2CH_2CH_2CH_2CH_3$, $COOCH_3$, $COOCH_2CH_3$ or $COOCH_2CH_2CH_3$.

Very few biphenols have been reported for their antiseptic or antioxidant purposes. 2,2′-Dihydroxy biphenyl and its derivatives were investigated for their fungicidal activity by Marsh and Butler (5), and 4,4′-dihydroxy biphenyl has been patented as an antioxidant (6).

In preliminary tests the dehydrobicatechols showed varying degrees of antioxidant activity by the Swift Stability Test using 0.005 per cent concentrations in lard. The alkyl substituted dehydrobicatechols showed good antioxidant activity on lard. On the *w/w* basis the activity of the alkyl substituted compounds decreased as the alkyl chain increased. A more rational comparison would be on a molar basis. The esters of

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† Abstracted in part from a thesis submitted by Akira [illegible] to the Faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
‡ Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.
§ The authors wish to thank Dr. K. K. Chen and the Bacteriology Department of Eli Lilly and Company for the antiseptic tests.

dehydrodiprotocatechuic acid showed very little activity. The results of the test are shown in Table I.

The results of the preliminary antiseptic test upon *Streptococcus viridans* using a synthetic chemical medium consisting of amino acids, vitamins, sugar, and salts are tabulated in Table II. Further investigation upon 2,2′,3,3′-tetrahydroxy-5,5′-dimethyl biphenyl; 2,2′,3,3′-tetrahydroxy-5,5′-di-*n*-propyl biphenyl; and di-*n*-propyl ester of 2,2′,3,3′-tetrahydroxy-5,5′-dicarboxy biphenyl showed phenol coefficients on *Streptococcus viridans* of 1600, 800 and 3200, respectively. These three most active compounds showed no activity against *Staphylococcus aureus* and *Bacillus typhosus* using concentrations as great as 1:50,000 in beef extract medium.

The reason for the great activity originally observed and apparent inertness in further experiments therefore was sought. Using the chemically defined medium, it was found that 1 per cent blood serum prevented the bacteriostatic action of these compounds against *Streptococcus viridans*. Inositol had no effect although it had slight structural similarity to these compounds.

TABLE I

0.005% of Compound	Hr. for Rancidity	Protective Index
2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl	51	6.0
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-propyl Biphenyl	35	4.1
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl	28	3.3
Dimethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl[a]	10	1.2
Diethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl[a]	15.5	1.8
Di-*n*-propyl Ester of 2,2′,-3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	12	1.4
Nordihydroguaiaretic Acid	36	4.2
Control	8.5	...

[a] Five milligrams of the compound were first dissolved in 0.5 cc. of propylene glycol, and the solution then added to the melted lard.

TABLE II

	No Growth	Growth
2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl	1:1,280,000	...
2,2′3,3′-Tetrahydroxy-5,5′di-*n*-propyl Biphenyl	1:1,280,000	...
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl	1:32,000	1:64,000
Diethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	1:80,000	1:160,000
Di-*n*-propyl Ester of 2,2′,-3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	1:1,280,000	...
Phenol	1:1000	1:5000

The reversal by such a small concentration of serum would indicate that they are not of value for sterilization of the skin, wounds, etc. The three most active compounds were also tested against influenza virus, *Streptococcus hemolyticus*, and tetanus toxin *in vivo*. The compounds were ineffective.

EXPERIMENTAL

Dehydrodieugenol or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-diallyl Biphenyl

Dehydrodieugenol previously has been prepared by a procedure that proved very unsatisfactory. The following procedure was devised and gave satisfactory results.

Twenty grams of eugenol was dissolved in 16 L. of distilled water, and to it was added, dropwise, a ferric chloride solution (33 Gm. of $FeCl_3 6H_2O$ in 250 cc. of water). The mixture was subjected to vigorous mechanical agitation during the addition of ferric chloride, which required approximately two hours. The agitation was continued for four hours, and a brown precipitate, which was obtained, was collected by filtration. About 13 Gm. of crude dehydrodieugenol was obtained. The precipitate was dissolved in 100 cc. of ether and then treated with 5% sodium hydroxide solution until no further precipitation occurred. The entire mixture was heated on a steam bath to remove the ether. The resultant mixture was filtered, and the precipitate was washed with 5% sodium hydroxide solution until the color of the washings became light yellow. Approximately 1500 cc. of 5% sodium hydroxide was necessary. The free phenol was liberated from the sodium salt by boiling the salt in 200 cc. of 10% hydrochloric acid. The mixture was cooled, and the precipitate filtered and washed with water. The collected product was then dried and extracted in a Soxhlet Extractor with petroleum ether (Skelly B) Dehydrodieugenol was only slightly soluble in petroleum ether, and upon prolonged extraction the dehydrodieugenol separated from petroleum ether in the flask. The solvent was decanted, and 10.5 Gm. of a semipure product which possessed a slight yellow color was obtained. This product was crystallized from 95% alcohol after partial decolorization with charcoal. An over-all yield of 30% dehydrodieugenol having a melting point of 101–103° (reported 106°) was obtained. This product was used for the next reaction. Through repeated recrystallizations it was possible to raise the melting point to 104–106°.

Tetrahydrodehydrodieugenol or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-di-*n*-propyl Biphenyl

Hydrogenation of the side chains of dehydrodieugenol was readily accomplished along the lines used by Erdtman. Four grams of dehydrodieugenol was dissolved in 350 cc. of 95% alcohol in a thick-walled bottle, and to it was added 0.1 Gm. of platinum oxide. The reaction bottle was connected to the Parr hydrogenation apparatus. After the introduction of 30 pounds of hydrogen, the bottle was shaken for one hour at room temperature although the hydrogenation appeared to be complete in ten minutes. The reduction product precipitated out. The content of the bottle was trans-

ferred into a beaker and heated to dissolve the reduced compound. The hot solution was filtered to remove the catalyst, and upon cooling tetrahydrodehydrodieugenol crystallized out from the filtrate. Three grams of product melting at 148–150° (reported 152°) was obtained.

By using the mother liquor as a solvent for the subsequent reduction of a second quantity of dehydrodieugenol, hydrogenation became slower and less complete. However, if the reaction mixture was heated to about 60° and then hydrogenated as before, a nearly quantitative amount of tetrahydrodehydrodieugenol was obtained. The mother liquor became slightly colored on repeated use, so it was decolorized with charcoal before each hydrogenation.

Nortetrahydrodehydrodieugenol or 2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-propyl Biphenyl

Five grams of tetrahydrodehydrodieugenol, 25 cc. of glacial acetic acid, 5 cc. of acetic anhydride, and 25 cc. of 48% hydrobromic acid were refluxed for two hours. The reaction mixture was allowed to cool and poured into 250 cc. of water. The reaction flask was rinsed with water and the whole combined. The precipitate that was collected by filtration amounted to 4.0 g. of crude nortetrahydrodehydrodieugenol. This compound was crystallized several times from dilute alcohol containing a small amount of sodium hydrosulfite. The yield was 2.5 Gm. or 59.3% of the theoretical. Melting point, 149–150°.

Anal.—Calcd. for $C_{18}H_{22}O_4$: C, 71.50; H, 7.34. Found: C, 71.74; H, 7.44.

The tetra-acetate was prepared by refluxing nortetrahydrodehydrodieugenol with pyridine-acetic anhydride solution. Melting point, 82.5–84.5°.

Anal.—Calcd. for $C_{26}H_{30}O_8$: C, 66.37; H, 6.42. Found: C, 66.56; H, 6.63.

Dehydrodivanillin or 2,2′dihydroxy-3,3′-dimethoxy-5,5′-dialdoxy Biphenyl

Dehydrodivanillin was prepared according to the directions of Elbs and Lerch (7) with slight modification in the purification. The crude washed product was suspended in 3 L. of boiling water, and the product was dissolved by the addition of 5% sodium hydroxide solution. Five per cent hydrochloric acid was added to this boiling solution to precipitate dehydrodivanillin. The precipitate was filtered and washed with 2 L. of boiling water. After repeating this purification process once more, a light gray product decomposing at 297–300° (reported 303–305°) was obtained in about 82% yield. This product was sufficiently pure for the preparation of dehydrodicreosol and dehydrodiprotocatachuic acid.

The purification method of Elbs and Lerch (7) did not raise the decomposition temperature. By Erdtman's purification method (8), a product decomposing at 300–303° was obtained.

The decomposition temperature for this compound was determined by preheating the melting point bath to 290° and by heating the bath at the rate of three degrees per minute.

Dehydrodiprotocatechuic Acid or 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl

The procedure of Tiemann (9) was used for the preparation of dehydrodiprotocatechuic acid. A yield of 6.2 Gm. of a light pink product was obtained from 10 Gm. of dehydrodivanillin. It decomposed at 306–310° (reported over 300°). This compound was difficultly soluble in the common polar and nonpolar solvents.

Di-*n*-propyl ester of dehydrodiprotocatechuic Acid

Two grams of dehydrodiprotocatechuic acid was suspended in 75 cc. of *n*-propyl alcohol which had been dried over "Drierite." Upon saturating the chilled suspension with dry hydrogen chloride the mixture became a pasty mass. This mixture was heated under a reflux until the solution was effected, which required approximately one hour, and the heating was continued for another hour. Excess alcohol and hydrogen chloride were removed under reduced pressure on the water pump, and 100 cc. of water was added to the residue. The product, collected by filtration, was shaken intermittently for an hour with 100 cc. of 5% sodium bicarbonate solution. The mixture was filtered and the solid residue washed with water. Two crystallizations from dilute propyl alcohol gave 1.6 Gm. or about 62.5% of a product that melted at 220–222°. A sample for analysis was recrystallized several times from dilute propyl alcohol. Light pink crystals melting at 221.5–223° were obtained.

Anal.—Calcd. for $C_{20}H_{22}O_8$: C, 61.53; H, 5.68. Found: C, 61.65; H, 6.03.

A tetra-acetate was prepared as follows: A solution of 0.3 Gm. of di-*n*-propyl ester of dehydrodiprotocatechuic acid in 5 cc. of acetic anhydride and 10 cc. of dry pyridine was refluxed for two hours, and then cooled to room temperature. The solution was poured into 50 cc. of ice water with constant stirring. The tetra-acetyl derivative which had separated was filtered, washed with 50 cc. of water, and then shaken for an hour with 25 cc. of 1% sodium hydroxide solution. The insoluble product was filtered and washed with water until the washings were no longer alkaline. Upon two crystallizations from dilute propyl alcohol a tetra-acetyl derivative melting at 105–107° was obtained.

Anal.—Calcd. for $C_{28}H_{30}O_{12}$: C, 60.21; H, 5.41. Found: C, 60.34; H, 5.75.

Diethyl ester of dehydrodiprotocatechuic Acid

Two grams of dehydrodiprotocatechuic acid was suspended in 75 cc. of absolute ethyl alcohol, and the ester was prepared according to the procedure used in the preparation of the propyl ester. A yield of 1.1 Gm. or 46.5% of white crystalline product melting at 257–259° was obtained after three crystallizations from dilute ethyl alcohol. The sample for analysis had a melting point of 259.5–260.5°.

Anal.—Calcd. for $C_{18}H_{18}O_8$: C, 59.67; H, 5.01; EtO, 24.86. Found: C, 59.23; H, 5.10, EtO, 24.69, 24.67.

The tetra-acetate was prepared. Melting point, 144–146°.

Anal.—Calcd. for $C_{26}H_{26}O_{12}$: C, 58.86; H, 4.94. Found: C, 59.09; H, 5.18.

Dimethyl ester of Dehydrodiprotocatechuic Acid

The dimethyl ester of dehydrodiprotocatechuic acid was prepared according to the procedure used in the preparation of the propyl ester. From 2 Gm. of dehydrodiprotocatechuic acid, 1.6 Gm. (73.4% yield) of a product was isolated. This ester was crystallized from dilute methyl alcohol and it had a melting point of 286–288°.

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Twenty grams of eugenol was dissolved in 16 L. c distilled water, and to it was added, dropwise, ferric chloride solution (33 Gm. of $FeCl_3 6H_2O$ i 250 cc. of water). The mixture was subjected t vigorous mechanical agitation during the additio of ferric chloride, which required approximately tw hours. The agitation was continued for four hours and a brown precipitate, which was obtained, wa collected by filtration. About 13 Gm. of crud dehydrodieugenol was obtained. The precipitat was dissolved in 100 cc. of ether and then treate with 5% sodium hydroxide solution until no furthe precipitation occurred. The entire mixture wa heated on a steam bath to remove the ether. Th resultant mixture was filtered, and the precipitat was washed with 5% sodium hydroxide solutio until the color of the washings became light yellow Approximately 1500 cc. of 5% sodium hydroxide was necessary. The free phenol was liberated from the sodium salt by boiling the salt in 200 cc. of 10% hydrochloric acid. The mixture was cooled, and the precipitate filtered and washed with water. The collected product was then dried and extracted in a Soxhlet Extractor with petroleum ether (Skelly B) Dehydrodieugenol was only slightly soluble in petroleum ether, and upon prolonged extraction the dehydrodieugenol separated from petroleum ether in the flask. The solvent was decanted, and 10.5 Gm. of a semipure product which possessed a slight yellow color was obtained. This product was crystallized from 95% alcohol after partial decolorization with charcoal. An over-all yield of 30% dehydrodieugenol having a melting point of 101–103° (reported 106°) was obtained. This product was used for the next reaction. Through repeated recrystallizations it was possible to raise the melting point to 104–106°.

Tetrahydrodehydrodieugenol or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-di-*n*-propyl Biphenyl

Hydrogenation of the side chains of dehydrodieugenol was readily accomplished along the lines used by Erdtman. Four grams of dehydrodieugenol was dissolved in 350 cc. of 95% alcohol in a thick-walled bottle, and to it was added 0.1 Gm. of platinum oxide. The reaction bottle was connected to the Parr hydrogenation apparatus. After the introduction of 30 pounds of hydrogen, the bottle was shaken for one hour at room temperature although the hydrogenation appeared to be complete in ten minutes. The reduction product precipitated out. The content of the bottle was trans-

ferred into a beaker and heated to dissolve the reduced compound. The hot solution was filtered to remove the catalyst, and upon cooling tetrahydrodehydrodieugenol crystallized out from the filtrate. Three grams of product melting at 148–150° (reported 152°) was obtained.

By using the mother liquor as a solvent for the subsequent reduction of a second quantity of dehydrodieugenol, hydrogenation became slower and less complete. However, if the reaction mixture was heated to about 60° and then hydrogenated as before, a nearly quantitative amount of tetrahydrodehydrodieugenol was obtained. The mother liquor became slightly colored on repeated use, so it was decolorized with charcoal before each hydrogenation.

Nortetrahydrodehydrodieugenol or 2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-propyl Biphenyl

Five grams of tetrahydrodehydrodieugenol, 25 cc. of glacial acetic acid, 5 cc. of acetic anhydride, and 25 cc. of 48% hydrobromic acid were refluxed for two hours. The reaction mixture was allowed to cool and poured into 250 cc. of water. The reaction flask was rinsed with water and the whole combined. The precipitate that was collected by filtration amounted to 4.0 g. of crude nortetrahydrodehydrodieugenol. This compound was crystallized several times from dilute alcohol containing a small amount of sodium hydrosulfite. The yield was 2.5 Gm. or 59.3% of the theoretical. Melting point, 149–150°.

Anal.—Calcd. for $C_{18}H_{22}O_4$: C, 71.50; H, 7.34. Found: C, 71.74; H, 7.44.

The tetra-acetate was prepared by refluxing nortetrahydrodehydrodieugenol with pyridine-acetic anhydride solution. Melting point, 82.5–84.5°.

Anal.—Calcd. for $C_{26}H_{30}O_8$: C, 66.37; H, 6.42. Found: C, 66.56; H, 6.63.

Dehydrodivanillin or 2,2′dihydroxy-3,3′-dimethoxy-5,5′-dialdoxy Biphenyl

Dehydrodivanillin was prepared according to the directions of Elbs and Lerch (7) with slight modification in the purification. The crude washed product was suspended in 3 L. of boiling water, and the product was dissolved by the addition of 5% sodium hydroxide solution. Five per cent hydrochloric acid was added to this boiling solution to precipitate dehydrodivanillin. The precipitate was filtered and washed with 2 L. of boiling water. After repeating this purification process once more, a light gray product decomposing at 297–300° (reported 303–305°) was obtained in about 82% yield. This product was sufficiently pure for the preparation of dehydrodicreosol and dehydrodiprotocatachuic acid.

The purification method of Elbs and Lerch (7) did not raise the decomposition temperature. By Erdtman's purification method (8), a product decomposing at 300–303° was obtained.

The decomposition temperature for this compound was determined by preheating the melting point bath to 290° and by heating the bath at the rate of three degrees per minute.

Dehydrodiprotocatechuic Acid or 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl

The procedure of Tiemann (9) was used for the preparation of dehydrodiprotocatechuic acid. A yield of 6.2 Gm. of a light pink product was obtained from 10 Gm. of dehydrodivanillin. It decomposed at 306–310° (reported over 300°). This compound was difficultly soluble in the common polar and nonpolar solvents.

Di-*n*-propyl ester of dehydrodiprotocatechuic Acid

Two grams of dehydrodiprotocatechuic acid was suspended in 75 cc. of *n*-propyl alcohol which had been dried over "Drierite." Upon saturating the chilled suspension with dry hydrogen chloride the mixture became a pasty mass. This mixture was heated under a reflux until the solution was effected, which required approximately one hour, and the heating was continued for another hour. Excess alcohol and hydrogen chloride were removed under reduced pressure on the water pump, and 100 cc. of water was added to the residue. The product, collected by filtration, was shaken intermittently for an hour with 100 cc. of 5% sodium bicarbonate solution. The mixture was filtered and the solid residue washed with water. Two crystallizations from dilute propyl alcohol gave 1.6 Gm. or about 62.5% of a product that melted at 220–222°. A sample for analysis was recrystallized several times from dilute propyl alcohol. Light pink crystals melting at 221.5–223° were obtained.

Anal.—Calcd. for $C_{20}H_{22}O_8$: C, 61.53; H, 5.68. Found: C, 61.65; H, 6.03.

A tetra-acetate was prepared as follows: A solution of 0.3 Gm. of di-*n*-propyl ester of dehydrodiprotocatechuic acid in 5 cc. of acetic anhydride and 10 cc. of dry pyridine was refluxed for two hours, and then cooled to room temperature. The solution was poured into 50 cc. of ice water with constant stirring. The tetra-acetyl derivative which had separated was filtered, washed with 50 cc. of water, and then shaken for an hour with 25 cc. of 1% sodium hydroxide solution. The insoluble product was filtered and washed with water until the washings were no longer alkaline. Upon two crystallizations from dilute propyl alcohol a tetra-acetyl derivative melting at 105–107° was obtained.

Anal.—Calcd. for $C_{28}H_{30}O_{12}$: C, 60.21; H, 5.41. Found: C, 60.34; H, 5.75.

Diethyl ester of dehydrodiprotocatechuic Acid

Two grams of dehydrodiprotocatechuic acid was suspended in 75 cc. of absolute ethyl alcohol, and the ester was prepared according to the procedure used in the preparation of the propyl ester. A yield of 1.1 Gm. or 46.5% of white crystalline product melting at 257–259° was obtained after three crystallizations from dilute ethyl alcohol. The sample for analysis had a melting point of 259.5–260.5°.

Anal.—Calcd. for $C_{18}H_{18}O_8$: C, 59.67; H, 5.01; EtO, 24.86. Found: C, 59.23; H, 5.10; EtO, 24.69, 24.67.

The tetra-acetate was prepared. Melting point, 144–146°.

Anal.—Calcd. for $C_{26}H_{26}O_{12}$: C, 58.86; H, 4.94. Found: C, 59.09; H, 5.18.

Dimethyl ester of Dehydrodiprotocatechuic Acid

The dimethyl ester of dehydrodiprotocatechuic acid was prepared according to the procedure used in the preparation of the propyl ester. From 2 Gm. of dehydrodiprotocatechuic acid, 1.6 Gm. (73.4% yield) of a product was isolated. This ester was crystallized from dilute methyl alcohol and it had a melting point of 286–288°.

dehydrodiprotocatechuic acid showed very little activity. The results of the test are shown in Table I.

The results of the preliminary antiseptic test upon *Streptococcus viridans* using a synthetic chemical medium consisting of amino acids, vitamins, sugar, and salts are tabulated in Table II. Further investigation upon 2,2′,3,3′-tetrahydroxy-5,5′-dimethyl biphenyl; 2,2′,3,3′-tetrahydroxy-5,5′-di-*n*-propyl biphenyl; and di-*n*-propyl ester of 2,2′,3,3′-tetrahydroxy-5,5′-dicarboxy biphenyl showed phenol coefficients on *Streptococcus viridans* of 1600, 800 and 3200, respectively. These three most active compounds showed no activity against *Staphylococcus aureus* and *Bacillus typhosus* using concentrations as great as 1:50,000 in beef extract medium.

The reason for the great activity originally observed and apparent inertness in further experiments therefore was sought. Using the chemically defined medium, it was found that 1 per cent blood serum prevented the bacteriostatic action of these compounds against *Streptococcus viridans*. Inositol had no effect although it had slight structural similarity to these compounds.

TABLE I

0.005% of Compound	Hr. for Rancidity	Protective Index
2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl	51	6.0
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-propyl Biphenyl	35	4.1
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl	28	3.3
Dimethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl[a]	10	1.2
Diethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl[a]	15.5	1 8
Di-*n*-propyl Ester of 2,2′,-3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	12	1.4
Nordihydroguaiaretic Acid	36	4.2
Control	8.5	...

[a] Five milligrams of the compound were first dissolved in 0.5 cc. of propylene glycol, and the solution then added to the melted lard.

TABLE II

	No Growth	Growth
2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl	1:1,280,000	...
2,2′3,3′-Tetrahydroxy-5,5′di-*n*-propyl Biphenyl	1:1,280,000	...
2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl	1:32,000	1:64,000
Diethyl Ester of 2,2′,3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	1:80,000	1:160,000
Di-*n*-propyl Ester of 2,2′,-3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl	1:1,280,000	...
Phenol	1:1000	1:5000

The reversal by such a small concentration of serum would indicate that they are not of value for sterilization of the skin, wounds, etc. The three most active compounds were also tested against influenza virus, *Streptococcus hemolyticus*, and tetanus toxin *in vivo*. The compounds were ineffective.

EXPERIMENTAL

Dehydrodieugenol or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-diallyl Biphenyl

Dehydrodieugenol previously has been prepared by a procedure that proved very unsatisfactory. The following procedure was devised and gave satisfactory results.

Twenty grams of eugenol was dissolved in 16 L. of distilled water, and to it was added, dropwise, a ferric chloride solution (33 Gm. of $FeCl_3 6H_2O$ in 250 cc. of water). The mixture was subjected to vigorous mechanical agitation during the addition of ferric chloride, which required approximately two hours. The agitation was continued for four hours, and a brown precipitate, which was obtained, was collected by filtration. About 13 Gm. of crude dehydrodieugenol was obtained. The precipitate was dissolved in 100 cc. of ether and then treated with 5% sodium hydroxide solution until no further precipitation occurred. The entire mixture was heated on a steam bath to remove the ether. The resultant mixture was filtered, and the precipitate was washed with 5% sodium hydroxide solution until the color of the washings became light yellow. Approximately 1500 cc. of 5% sodium hydroxide was necessary. The free phenol was liberated from the sodium salt by boiling the salt in 200 cc. of 10% hydrochloric acid. The mixture was cooled, and the precipitate filtered and washed with water. The collected product was then dried and extracted in a Soxhlet Extractor with petroleum ether (Skelly B) Dehydrodieugenol was only slightly soluble in petroleum ether, and upon prolonged extraction the dehydrodieugenol separated from petroleum ether in the flask. The solvent was decanted, and 10.5 Gm. of a semipure product which possessed a slight yellow color was obtained. This product was crystallized from 95% alcohol after partial decolorization with charcoal. An over-all yield of 30% dehydrodieugenol having a melting point of 101–103° (reported 106°) was obtained. This product was used for the next reaction. Through repeated recrystallizations it was possible to raise the melting point to 104–106°.

Tetrahydrodehydrodieugenol or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-di-*n*-propyl Biphenyl

Hydrogenation of the side chains of dehydrodieugenol was readily accomplished along the lines used by Erdtman. Four grams of dehydrodieugenol was dissolved in 350 cc. of 95% alcohol in a thick-walled bottle, and to it was added 0.1 Gm. of platinum oxide. The reaction bottle was connected to the Parr hydrogenation apparatus. After the introduction of 30 pounds of hydrogen, the bottle was shaken for one hour at room temperature although the hydrogenation appeared to be complete in ten minutes. The reduction product precipitated out. The content of the bottle was trans-

ferred into a beaker and heated to dissolve the reduced compound. The hot solution was filtered to remove the catalyst, and upon cooling tetrahydrodehydrodieugenol crystallized out from the filtrate. Three grams of product melting at 148–150° (reported 152°) was obtained.

By using the mother liquor as a solvent for the subsequent reduction of a second quantity of dehydrodieugenol, hydrogenation became slower and less complete. However, if the reaction mixture was heated to about 60° and then hydrogenated as before, a nearly quantitative amount of tetrahydrodehydrodieugenol was obtained. The mother liquor became slightly colored on repeated use, so it was decolorized with charcoal before each hydrogenation.

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Di-*n*-propyl ester of dehydrodiprotocatechuic Acid

Two grams of dehydrodiprotocatechuic acid was suspended in 75 cc. of *n*-propyl alcohol which had been dried over "Drierite." Upon saturating the chilled suspension with dry hydrogen chloride the mixture became a pasty mass. This mixture was heated under a reflux until the solution was effected, which required approximately one hour, and the heating was continued for another hour. Excess alcohol and hydrogen chloride were removed under reduced pressure on the water pump, and 100 cc. of water was added to the residue. The product, collected by filtration, was shaken intermittently for an hour with 100 cc. of 5% sodium bicarbonate solution. The mixture was filtered and the solid residue washed with water. Two crystallizations from dilute propyl alcohol gave 1.6 Gm. or about 62.5% of a product that melted at 220–222°. A sample for analysis was recrystallized several times from dilute propyl alcohol. Light pink crystals melting at 221.5–223° were obtained.

Anal.—Calcd. for $C_{20}H_{22}O_8$: C, 61.53; H, 5.68. Found: C, 61.65; H, 6.03.

A tetra-acetate was prepared as follows: A solution of 0.3 Gm. of di-*n*-propyl ester of dehydrodiprotocatechuic acid in 5 cc. of acetic anhydride and 10 cc. of dry pyridine was refluxed for two hours, and then cooled to room temperature. The solution was poured into 50 cc. of ice water with constant stirring. The tetra-acetyl derivative which had separated was filtered, washed with 50 cc. of water, and then shaken for an hour with 25 cc. of 1% sodium hydroxide solution. The insoluble product was filtered and washed with water until the washings were no longer alkaline. Upon two crystallizations from dilute propyl alcohol a tetra-acetyl derivative melting at 105–107° was obtained.

Anal.—Calcd. for $C_{28}H_{30}O_{12}$: C, 60.21; H, 5.41. Found: C, 60.34; H, 5.75.

Diethyl ester of dehydrodiprotocatechuic Acid

Two grams of dehydrodiprotocatechuic acid was suspended in 75 cc. of absolute ethyl alcohol, and the ester was prepared according to the procedure used in the preparation of the propyl ester. A yield of 1.1 Gm. or 46.5% of white crystalline product melting at 257–259° was obtained after three crystallizations from dilute ethyl alcohol. The sample for analysis had a melting point of 259.5–260.5°.

Anal.—Calcd. for $C_{18}H_{18}O_8$: C, 59.67; H, 5.01; EtO, 24.86. Found: C, 59.23; H, 5.10; EtO, 24.69, 24.67.

The tetra-acetate was prepared. Melting point, 144–146°.

Anal.—Calcd. for $C_{26}H_{26}O_{12}$: C, 58.86; H, 4.94. Found: C, 59.09; H, 5.18.

Dimethyl ester of Dehydrodiprotocatechuic Acid

The dimethyl ester of dehydrodiprotocatechuic acid was prepared according to the procedure used in the preparation of the propyl ester. From 2 Gm. of dehydrodiprotocatechuic acid, 1.6 Gm. (73.4% yield) of a product was isolated. This ester was crystallized from dilute methyl alcohol and it had a melting point of 286–288°.

Anal.—Calcd. for $C_{16}H_{14}O_8$: C, 57.49; H, 4.22; MeO, 18.56. Found: C, 57.38; H, 4.57; MeO, 18.68, 18.57.

The tetra-acetyl derivative was prepared as described above. Melting point, 134–136°.

Anal.—Calcd. for $C_{24}H_{22}O_{12}$: C, 57.37; H, 4.41. Found: C, 57.01; H, 4.61.

Dehydrodicreosol or 2,2′-Dihydroxy-3,3′-dimethoxy-5′,5′-dimethyl Biphenyl

A. By the Oxidative Coupling of Creosol.—Six cubic centimeters of creosol was dissolved in 1000 cc. of distilled water and the solution chilled to 5°. Ten cubic centimeters of ferric chloride solution (9 Gm. of $FeCl_3 \cdot 6H_2O$ in 100 cc. of water) was added slowly with constant shaking, and the mixture was allowed to stand at room temperature for one week. The precipitated produced was collected by filtration which amounted to 0.7 Gm. of a rather crude sticky product. Two crystallizations from dilute alcohol produced 0.2 Gm. of a white crystalline dehydrodicreosol, melting at 130–132°.

B. By the Reduction of Dehydrodivanillin.—Ten grams of dehydrodivanillin, 125 cc. of concentrated hydrochloric acid, 125 cc. of 95% alcohol, 25 Gm. of amalgamated zinc dust, and 75 cc. of water were heated under the reflux for an hour. Then a second 25 Gm. of amalgamated zinc dust was added, and the mixture was refluxed for an hour after solution was effected. The time required for solution varied, but it was usually complete in two hours. The mixture was filtered while hot to remove excess amalgamated zinc. Upon cooling a precipitate was obtained, which was collected by filtration. An additional precipitate resulted upon the addition of water to the filtrate. About 9 Gm. of crude product having a melting range of 107–115° was obtained. This product was purified by fractional solution in dilute alcohol. The concentration of alcohol was held to a minimum so as to dissolve the least amount of oxidized reddish tarry material, which was removed by filtration of the hot solution. The process was repeated several times. After the major portion of the tarry material had been removed by this process, the product was recrystallized from a higher concentration of alcohol, producing 2.5 Gm. of light pink crystals which melted at 127–129°. The sample for analysis was repeatedly recrystallized from dilute alcohol until a white product was obtained. Melting point, 130–132°.

Anal.—Calcd. for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61; MeO, 22.62. Found: C, 70.05; H, 6.91; MeO, 22.47, 22.59.

The diacetyl derivative was prepared by the usual method. Melting point, 162–163°.

Anal.—Calcd. for $C_{20}H_{22}O_6$: C, 67.02; H, 6.19. Found: C, 66.67; H, 6.56.

The dibenzoate was prepared. Melting point, 193–195°.

Anal.—Calcd. for $C_{30}H_{26}O_6$: C, 74.67; H, 5.43. Found: C, 74.70; H, 5.78.

Nordehydrodicreosol or 2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl

Three grams of dehydrodicreosol was demethylated with the glacial acetic acid-hydrobromic acid mixture previously described to yield 0.7 Gm. of a light pink crystalline product. Melting point, 220–222°.

Anal.—Calcd. for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 68.21; H, 6.02.

The tetra-acetate was prepared as previously described. Melting point, 110–112°.

Anal.—Calcd. for $C_{22}H_{22}O_8$: C, 63.76; H, 5.35. Found: C, 63.58; H, 5.49.

Guaiacyl Valerate

Miller, Hartung, Rock, and Crossley (10) reported a general procedure for the preparation of guaiacol esters. This procedure was used to prepare guaiacyl valerate. Excess thionyl chloride was removed from the reaction mixture by distillation rather than by washing with a bicarbonate solution. The fraction distilling between 255–265° was collected and used for the next reaction. A yield of 80% of the ester was obtained.

4-*n*-Vateryl Guaiacol

4-*n*-Vateryl guaiacol was prepared according to the procedure of Coulthard and co-workers (11) with some modifications. No attempt was made to separate a portion of the aluminum chloride complex that had separated from the reaction mixture. To the reaction mixture 500 cc. of hydrochloric acid (1:1) was added slowly, and the mixture was warmed on the steam bath to dissolve all the precipitate. The nitrobenzene layer was separated and washed twice with 100-cc. portions of water. The nitrobenzene solution was then steam distilled to remove nitrobenzene, and the remaining oil was distilled under 15 mm. pressure. The fraction distilling between 190–200° was collected and placed in the refrigerator overnight. The solidified mass was then filtered on a Büchner funnel to remove a yellow oil which accompanied the product. Crystallization from 95% alcohol yielded 30.3 Gm. of 4-*n*-vateryl guaiacol that melted at 59–61° (reported 60–62°). An additional quantity of 6 Gm. was recovered from the mother liquor.

4-*n*-Amyl Guaiacol

The carbonyl group of 4-*n*-vateryl guaiacol was reduced by the Clemmensen method. Twenty grams of 4-*n*-vateryl guaiacol, 60 Gm. of amalgamated zinc dust, 120 cc. of hydrochloric acid (1:1), and 40 cc. of alcohol were heated under reflux for twelve hours. The reaction mixture was allowed to cool, and the excess amalgamated zinc dust was removed by filtration. The alcohol was distilled off on the steam bath, and the remaining liquid was extracted with 150 cc. of ether. The ether solution was then washed with 25 cc. of water and dried over anhydrous sodium sulfate. After distillation of the ether, there remained 14 Gm. of oil. This oil was distilled under 15 mm. of pressure, and the fraction distilling between 155–157° was collected. 4-*n*-amyl guaiacol thus obtained was colorless, but became light yellow upon standing.

Dehydrodi-(4-*n*-amyl Guaiacol) or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-di-*n*-amyl Biphenyl

Eight cubic centimeters of 4-*n*-amyl guaiacol was dissolved in 3000 cc. of 40% alcohol, and a solution of ferric chloride, prepared by dissolving 14 Gm. of $FeCl_3 \cdot 6H_2O$ in 100 cc. of 50% alcohol, was added through a dropping funnel with constant stirring. The addition required about three hours. The mixture was allowed to stand for twenty-four hours, and the product that precipitated was filtered. The precipitate was washed with 15 cc. of petroleum benzin (U. S. P.), which yielded 3.6 Gm. of light tan-colored product. After two crystallizations from

dilute alcohol 3 Gm. of dehydrodi-(4-*n*-amyl guaiacol) were obtained. Melting point, 114–116°.

Anal.—Calcd. for $C_{24}H_{34}O_4$: C, 74.58; H, 8.87; MeO, 16.07. Found: C, 74.36; H, 8.56; MeO, 16.03, 16.01.

The diacetate was prepared. Melting point, 70–73°.

Anal.—Calcd. for $C_{28}H_{38}O_6$: C, 71.46; H, 8.14. Found: C, 71.14; H, 8.00.

Nordehydrodi-(4-*n*-amyl Guaiacol) or 2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl

The demethylation of dehydrodi-(4-*n*-amyl guaiacol) was accomplished by the above-described method. From 3 Gm. of dehydrodi-(4-*n*-amyl guaiacol), 1.8 Gm. of nordehydrodi-(4-*n*-amyl guaiacol) melting at 146–148° was obtained.

Anal.—Calcd. for $C_{22}H_{30}O_4$: C, 73.71; H, 8.44. Found: C, 74.04; H 8.53.

The tetra-*p*-nitrobenzoate was prepared. Melting point, 152–154°.

Anal.—Calcd. for $C_{50}H_{42}O_{16}N_4$: C, 62.88; H, 4.43. Found: C, 63.00; H, 4.56.

Ferric Chloride Color Tests

Ferric chloride color tests were made on all substituted dehydrobiguaiacols and dehydrobicatechols which were prepared in this investigation. In most cases it was necessary to have a rather concentrated alcoholic solution of the compound before true color changes were noticeable. With ferric chloride test solution all the alcoholic solutions of the dehydrobicatechols gave a very transient green color, which immediately turned blue. On addition of sodium bicarbonate solution the color changed to a dark red. The alcoholic solution of dehydrobiguaiacols gave a green color with ferric chloride.

SUMMARY

The synthesis of six new 5,5′-disubstituted 2,2′,3,3′-tetrahydroxy biphenyls was carried out by the oxidative coupling of 4-substituted guaiacols and subsequent demethylation of the bis compounds or by some modifications thereof. In the alkyl substituted 2,2′,3,3′-tetrahydroxy biphenyls the methyl, *n*-propyl, and *n*-amyl compounds were prepared, and in the carboxylic acid substituted compounds (dehydrodiprotocatechuic acid) the methyl, ethyl, and *n*-propyl esters were prepared.

Antioxidant tests were made. The alkyl substituted dehydrobicatechols showed marked activity. On a weight-in-weight basis the activity decreased as the alkyl chain increased. The esters of dehydrodiprotocatechuic acid showed very little activity.

The dehydrobicatechols showed marked bacteriostatic activity when a synthetic chemical medium was used. The bacteriostatic activity was destroyed by 1 per cent blood serum.

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Notes

Note on the Determination of Estrone, Equilin, and Equilenin by Infrared Spectrophotometry*

By FREDERICK C. NACHOD, E. T. HINKEL, JR., and CALVIN ZIPPIN

THE SIMULTANEOUS DETERMINATION of the principal components of natural estrogenic materials by means of infrared spectra has been described recently by Carol, Molitor, and Haenni (1). They were able to show that the maxima at 10.45 μ, 10.88 μ, and 10.96 μ are characteristic for equilenin, estrone, and equilin, respectively, but at each wave length, the two other estrogens also contribute to the total observed absorption.

To allow for such contributions, the authors have suggested a method of successive approximations

* Received Sept. 13, 1948, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

Anal.—Calcd. for $C_{16}H_{14}O_8$: C, 57.49; H, 4.22; MeO, 18.56. Found: C, 57.38; H, 4.57; MeO, 18.68, 18.57.

The tetra-acetyl derivative was prepared as described above. Melting point, 134–136°.

Anal.—Calcd. for $C_{24}H_{22}O_{12}$: C, 57.37; H, 4.41. Found: C, 57.01; H, 4.61.

Dehydrodicreosol or 2,2′-Dihydroxy-3,3′-dimethoxy-5′,5′-dimethyl Biphenyl

A. By the Oxidative Coupling of Creosol.—Six cubic centimeters of creosol was dissolved in 1000 cc. of distilled water and the solution chilled to 5°. Ten cubic centimeters of ferric chloride solution (9 Gm. of $FeCl_3 \cdot 6H_2O$ in 100 cc. of water) was added slowly with constant shaking, and the mixture was allowed to stand at room temperature for one week. The precipitated produced was collected by filtration which amounted to 0.7 Gm. of a rather crude sticky product. Two crystallizations from dilute alcohol produced 0.2 Gm. of a white crystalline dehydrodicreosol, melting at 130–132°.

B. By the Reduction of Dehydrodivanillin.—Ten grams of dehydrodivanillin, 125 cc. of concentrated hydrochloric acid, 125 cc. of 95% alcohol, 25 Gm. of amalgamated zinc dust, and 75 cc. of water were heated under the reflux for an hour. Then a second 25 Gm. of amalgamated zinc dust was added, and the mixture was refluxed for an hour after solution was effected. The time required for solution varied, but it was usually complete in two hours. The mixture was filtered while hot to remove excess amalgamated zinc. Upon cooling a precipitate was obtained, which was collected by filtration. An additional precipitate resulted upon the addition of water to the filtrate. About 9 Gm. of crude product having a melting range of 107–115° was obtained. This product was purified by fractional solution in dilute alcohol. The concentration of alcohol was held to a minimum so as to dissolve the least amount of oxidized reddish tarry material, which was removed by filtration of the hot solution. The process was repeated several times. After the major portion of the tarry material had been removed by this process, the product was recrystallized from a higher concentration of alcohol, producing 2.5 Gm. of light pink crystals which melted at 127–129°. The sample for analysis was repeatedly recrystallized from dilute alcohol until a white product was obtained. Melting point, 130–132°.

Anal.—Calcd. for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61; MeO, 22.62. Found: C, 70.05; H, 6.91; MeO, 22.47, 22.59.

The diacetyl derivative was prepared by the usual method. Melting point, 162–163°.

Anal.—Calcd. for $C_{20}H_{22}O_6$: C, 67.02; H, 6.19. Found: C, 66.67; H, 6.56.

The dibenzoate was prepared. Melting point, 193–195°.

Anal.—Calcd. for $C_{30}H_{26}O_6$: C, 74.67; H, 5.43. Found: C, 74.70; H, 5.78.

Nordehydrodicreosol or 2,2′,3,3′-Tetrahydroxy-5,5′-dimethyl Biphenyl

Three grams of dehydrodicreosol was demethylated with the glacial acetic acid-hydrobromic acid mixture previously described to yield 0.7 Gm. of a light pink crystalline product. Melting point, 220–222°.

Anal.—Calcd. for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 68.21; H, 6.02.

The tetra-acetate was prepared as previously described. Melting point, 110–112°.

Anal.—Calcd. for $C_{22}H_{22}O_8$: C, 63.76; H, 5.35. Found: C, 63.58; H, 5.49.

Guaiacyl Valerate

Miller, Hartung, Rock, and Crossley (10) reported a general procedure for the preparation of guaiacol esters. This procedure was used to prepare guaiacyl valerate. Excess thionyl chloride was removed from the reaction mixture by distillation rather than by washing with a bicarbonate solution. The fraction distilling between 255–265° was collected and used for the next reaction. A yield of 80% of the ester was obtained.

4-*n*-Vateryl Guaiacol

4-*n*-Vateryl guaiacol was prepared according to the procedure of Coulthard and co-workers (11) with some modifications. No attempt was made to separate a portion of the aluminum chloride complex that had separated from the reaction mixture. To the reaction mixture 500 cc. of hydrochloric acid (1:1) was added slowly, and the mixture was warmed on the steam bath to dissolve all the precipitate. The nitrobenzene layer was separated and washed twice with 100-cc. portions of water. The nitrobenzene solution was then steam distilled to remove nitrobenzene, and the remaining oil was distilled under 15 mm. pressure. The fraction distilling between 190–200° was collected and placed in the refrigerator overnight. The solidified mass was then filtered on a Büchner funnel to remove a yellow oil which accompanied the product. Crystallization from 95% alcohol yielded 30.3 Gm. of 4-*n*-vateryl guaiacol that melted at 59–61° (reported 60–62°). An additional quantity of 6 Gm. was recovered from the mother liquor.

4-*n*-Amyl Guaiacol

The carbonyl group of 4-*n*-vateryl guaiacol was reduced by the Clemmensen method. Twenty grams of 4-*n*-vateryl guaiacol, 60 Gm. of amalgamated zinc dust, 120 cc. of hydrochloric acid (1:1), and 40 cc. of alcohol were heated under reflux for twelve hours. The reaction mixture was allowed to cool, and the excess amalgamated zinc dust was removed by filtration. The alcohol was distilled off on the steam bath, and the remaining liquid was extracted with 150 cc. of ether. The ether solution was then washed with 25 cc. of water and dried over anhydrous sodium sulfate. After distillation of the ether, there remained 14 Gm. of oil. This oil was distilled under 15 mm. of pressure, and the fraction distilling between 155–157° was collected. 4-*n*-amyl guaiacol thus obtained was colorless, but became light yellow upon standing.

Dehydrodi-(4-*n*-amyl Guaiacol) or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-di-*n*-amyl Biphenyl

Eight cubic centimeters of 4-*n*-amyl guaiacol was dissolved in 3000 cc. of 40% alcohol, and a solution of ferric chloride, prepared by dissolving 14 Gm. of $FeCl_3 \cdot 6H_2O$ in 100 cc. of 50% alcohol, was added through a dropping funnel with constant stirring. The addition required about three hours. The mixture was allowed to stand for twenty-four hours, and the product that precipitated was filtered. The precipitate was washed with 15 cc. of petroleum benzin (U. S. P.), which yielded 3.6 Gm. of light tan-colored product. After two crystallizations from

dilute alcohol 3 Gm. of dehydrodi-(4-*n*-amyl guaiacol) were obtained. Melting point, 114–116°.

Anal.—Calcd. for $C_{24}H_{34}O_4$: C, 74.58; H, 8.87; MeO, 16.07. Found: C, 74.36; H, 8.56; MeO, 16.03, 16.01.

The diacetate was prepared. Melting point, 70–73°.

Anal.—Calcd. for $C_{28}H_{38}O_6$: C, 71.46; H, 8.14. Found: C, 71.14; H, 8.00.

Nordehydrodi-(4-*n*-amyl Guaiacol) or 2,2′,3,3′-Tetrahydroxy-5,5′-di-*n*-amyl Biphenyl

The demethylation of dehydrodi-(4-*n*-amyl guaiacol) was accomplished by the above-described method. From 3 Gm. of dehydrodi-(4-*n*-amyl guaiacol), 1.8 Gm. of nordehydrodi-(4-*n*-amyl guaiacol) melting at 146–148° was obtained.

Anal.—Calcd. for $C_{22}H_{30}O_4$: C, 73.71; H, 8.44. Found: C, 74.04; H 8.53.

The tetra-*p*-nitrobenzoate was prepared. Melting point, 152–154°.

Anal.—Calcd. for $C_{50}H_{42}O_{16}N_4$: C, 62.88; H, 4.43. Found: C, 63.00; H, 4.56.

Ferric Chloride Color Tests

Ferric chloride color tests were made on all substituted dehydrobiguaiacols and dehydrobicatechols which were prepared in this investigation. In most cases it was necessary to have a rather concentrated alcoholic solution of the compound before true color changes were noticeable. With ferric chloride test solution all the alcoholic solutions of the dehydrobicatechols gave a very transient green color, which immediately turned blue. On addition of sodium bicarbonate solution the color changed to a dark red. The alcoholic solution of dehydrobiguaiacols gave a green color with ferric chloride.

SUMMARY

The synthesis of six new 5,5′-disubstituted 2,2′,3,3′-tetrahydroxy biphenyls was carried out by the oxidative coupling of 4-substituted guaiacols and subsequent demethylation of the bis compounds or by some modifications thereof. In the alkyl substituted 2,2′,3,3′-tetrahydroxy biphenyls the methyl, *n*-propyl, and *n*-amyl compounds were prepared, and in the carboxylic acid substituted compounds (dehydrodiprotocatechuic acid) the methyl, ethyl, and *n*-propyl esters were prepared.

Antioxidant tests were made. The alkyl substituted dehydrobicatechols showed marked activity. On a weight-in-weight basis the activity decreased as the alkyl chain increased. The esters of dehydrodiprotocatechuic acid showed very little activity.

The dehydrobicatechols showed marked bacteriostatic activity when a synthetic chemical medium was used. The bacteriostatic activity was destroyed by 1 per cent blood serum.

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Notes

Note on the Determination of Estrone, Equilin, and Equilenin by Infrared Spectrophotometry*

By FREDERICK C. NACHOD, E. T. HINKEL, JR., and CALVIN ZIPPIN

THE SIMULTANEOUS DETERMINATION of the principal components of natural estrogenic materials by means of infrared spectra has been described recently by Carol, Molitor, and Haenni (1). They were able to show that the maxima at 10.45 μ, 10.88 μ, and 10.96 μ are characteristic for equilenin, estrone, and equilin, respectively, but at each wave length, the two other estrogens also contribute to the total observed absorption.

To allow for such contributions, the authors have suggested a method of successive approximations

* Received Sept. 13, 1948, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

which appears cumbersome and time consuming. A procedure is suggested herein which avoids these approximations by solving three simultaneous equations (2).

PROCEDURE

Since there are three discrete maxima for the three estrogenic compounds, one can write three simultaneous equations. C_a denotes the concentration of equilenin, C_b the concentration of estrone, and C_c the concentration of equilin; and k_a, $k_b \ldots$, $k_b' \ldots$, k_b'', k_c'' indicate the proportionality constants which are ratios of optical density divided by concentration. Thus is written:

$$O.\,D._{10\cdot45} = k_aC_a + k_bC_b + k_cC_c \quad \text{(I)}$$
$$O.\,D._{10\cdot88} = k_a'C_a + k_b'C_b + k_c'C_c \quad \text{(II)}$$
$$O.\,D._{10\cdot96} = k_a''C_a + k_b''C_b + k_c''C_c \quad \text{(III)}$$

The equations (I), (II), and (III) can be solved for C_a, C_b, C_c after the k-values have been determined from suitable calibration charts. For example, taking data from the "working curves" of Fig. 3 of the paper by Carol, *et al.*, the nine finite k-values, e.g., the slopes of the curves can be inserted into these equations and the following is obtained:

$$O.\,D._{10\cdot45} = \mathit{0.0407}\ C_a + 0.0055\ C_b + 0.0097\ C_c \quad \text{(IV)}$$
$$O.\,D._{10\cdot88} = 0.0066\ C_a + \mathit{0.0531}\ C_b + 0.0165\ C_c \quad \text{(V)}$$
$$O.\,D._{10\cdot96} = 0.0068\ C_a + 0.0216\ C_b + \mathit{0.0374}\ C_c \quad \text{(VI)}$$

In the above, the major contributing terms, corresponding to the first approximation of Carol and collaborators, are italicized.

It is to be noted that all O. D. values are measurable and only three unknown quantities, C_a, C_b, and C_c, remain. By solving three simultaneous equations in the usual manner for C_a, C_b, and C_c, one obtains:

Concentration of equilenin:

$$C_a = 25.65\ O.\,D._{10\cdot45} + 0.03\ O.\,D._{10\cdot88} - 6.68\ O.\,D._{10\cdot96} \quad \text{(VII)}$$

Concentration of estrone:

$$C_b = 22.95\ O.\,D._{10\cdot88} - 2.12\ O.\,D._{10\cdot45} - 9.57\ O.\,D._{10\cdot96} \quad \text{(VIII)}$$

Concentration of equilin:

$$C_c = 33.48\ O.\,D._{10\cdot96} - 3.44\ O.\,D._{10\cdot45} - 13.26\ O.\,D._{10\cdot88} \quad \text{(IX)}$$

In a hypothetical example where C_a was assumed to be 6 mg./ml., C_b 4 mg./ml., and C_c 8 mg./ml., the expected optical densities at the three wave lengths were computed by adding the individual contributions of the three estrogens. Substituting these values into equations (VII), (VIII), and (IX) the calculated C-values were: C_a = 6.10 mg./ml. C_b = 3.95 mg./ml., and C_c = 7.94 mg./ml.

The k-values here reported are based on the experimental data of Carol and co-workers. Due to differences between instruments as well as possibly between calibration compounds, the numerical values may have to be revised slightly by other workers in the field, to fit their specific needs. However, the principle here employed will be applicable in all cases.

SUMMARY

The method of Carol and co-workers for the determination of estrogenic materials by successive approximations has been extended and subjected to mathematical treatment. By solving three simultaneous equations and by using numerical values for proportionality factors, the calculation of estrogenic materials can be simplified.

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Vitamins B_1. A Note on the Stability of Thiamine Hydrochloride in Aqueous Solution in the Presence of Iodine and Potassium Iodide*

By HUBERT W. MURPHY and JOHN M. GOODYEAR

DURING the investigation of the formation of a precipitate which was produced when Lugol's Solution was added to an elixir of thiamine hydrochloride made with sucrose and the absence of such a precipitate when the elixir was made with glucose, the stability of thiamine hydrochloride in such mixtures was studied simultaneously. The immediate formation of a precipitate apparently is prevented in the elixir made with glucose by absorption of the iodine on dextrins.

Since inquiry has been made with regard to the stability of thiamine hydrochloride in the presence of iodine and iodides and the opinion expressed (1) is at variance with our data, it would appear desirable to correct this concept.

EXPERIMENTAL

Typical data reproduced in Table I indicate that, within experimental error, the presence of iodine and

* Received July 17, 1948, from the Chemical Control Division, Eli Lilly and Company, Indianapolis 6, Ind.

iodides does not exert a destructive effect upon thiamine hydrochloride in aqueous solutions. This stability was studied within a *p*H range of 2.0 to 4.8. All samples were stored in amber glass bottles in diffused sunlight at room temperature which varied from 23° to 33°. The presence of ethyl alcohol in concentrations up to 15% had little or no effect upon the stability of the thiamine in these preparations.

TABLE I.—STABILITY OF THIAMINE HYDROCHLORIDE IN AQUEOUS PREPARATIONS CONTAINING IODINE AND POTASSIUM IODIDE

Preparation	Thiamine Hydrochloride Found Expressed as % of That Originally Present			
	1 Year	2 Years	3 Years	4 Years
A	..	86	82	85
B	..	88	88	86.5
C	92	88	87	86

PREPARATION A

Glucose	37.5 Gm.
Thiamine Hydrochloride	0.348 Gm.
Lugol's Solution	2.0 cc.
Water, Distilled, *q. s.* to make	100 cc.

PREPARATION B

Sucrose	25.0 Gm.
Thamine Hydrochloride	0.376 Gm.
Lugol's Solution	2.0 cc.
Water, Distilled, *q. s.* to make	100 cc.

PREPARATION C

Glucose	37.5 Gm
Thiamine Hydrochloride	0.40 Gm.
Hydrochloric Acid U.S.P. (*q. s.* to adjust *p*H to 3.0)	
Water, Dist., *q. s.* to make	100 cc.

The *p*H of each of the first two mixtures was approximately 2.20 and C was approximately 3.0 throughout the four-year period of observation. Assays were made by the standard U. S. P. thiochrome method. Blanks were normal and displayed no extraneous fluorescence. The assays recorded for the end of the four-year aging period were made by both the thiochrome method and the microbiological yeast fermentation method. Very close agreement in the results of both methods was observed.

CONCLUSIONS

The presence of glucose in an elixir of thiamine hydrochloride prevents the immediate formation of a precipitate upon the addition of 2% by volume of Lugol's Solution.

The stability of thiamine hydrochloride in aqueous solution within the *p*H range of 2.0 to 4.8 does not appear to be influenced by the presence of glucose as a replacement for sucrose or by ethyl alcohol in a concentration up to 15%. The presence of iodine and potassium iodide in concentrations equivalent to 2% of Lugol's Solution by volume does not exert a destructive effect on thiamine hydrochloride in aqueous solutions in the presence of glucose or sucrose with or without the addition of up to 15% alcohol within the *p*H range of 2.0 to 4.8. There should be no objections to combinations of iodine or iodides and thiamine hydrochloride in mixtures of this type.

REFERENCE

(1) THIS JOURNAL, *Pract. Pharm. Ed.*, 8, 379(1947).

Book Reviews

New and Nonofficial Remedies, 1948. Issued under the direction and supervision of the Council on Pharmacy and Chemistry of the American Medical Association. J. B. Lippincott Company, Philadelphia, 1948. li + 800 pp. 12 x 19 cm. Price $3.00.

New and Nonofficial Remedies is one of the most valuable books in the pharmacist's library. Although it is written for physicians, pharmacists have adopted it and follow the appearance of new editions with much interest.

As each year's issue of this handy book became available we noticed it was thicker than the previous one. The 1948 issue has become so large that the publishers may find it necessary to change the format somewhat. Because we appreciate the problem that the increase in size has brought and because we recognize some of the dilemmas facing the Council on Pharmacy and Chemistry, these few suggestions are ventured in the hope that they will help in planning future editions.

First there is the matter of scope. The real value, it seems to us, in this excellent work lies in the *newness* and the *nonofficial* character of the drugs.

The Council very wisely has a rule to eliminate drugs which have been official for over twenty years, although we are inclined to wonder if the time should not be shortened now that the U. S. P. and the N. F. are revised every five years. Nevertheless in view of this rule, one is surprised to find agar, barbital, barium sulfate, carbromal, ephedrine, petrolatum, phenobarbital, quinidine sulfate, quinine sulfate, silver nitrate, theophylline, thymol iodide, and others still present. Many of the nonofficial drugs which are over twenty years old are still recognized, too—witness Afenil, bismuth tribromophenate, metacresyl acetate, Digalen, ethyl salicylate, ferrous lactate, Orthoform, scarlet red sulfonate, sodium peroxide, and many other drugs.

In making a hasty count, it was noted that nearly half of the drugs in the 1948 edition of N. N. R. are at present official in either the U. S. P. or the N. F. In the category of local anti-infective drugs, 23 are nonofficial, 19 in the N. F. and 9 in the U. S. P. In the systemic anti-infective group, 11 are nonofficial, 4 are N. F. and 18 are U. S. P. Among the autonomic drugs, 18 are nonofficial and 10 are in the U. S. P. Eighteen hormones and synthetic

substitutes are nonofficial and 9 are in the U. S. P.; 11 vitamin drugs are nonofficial and 15 are in the U. S. P., etc.

Perhaps one of the most difficult achievements of any work pertaining to drug standards is consistency. N. N. R. 1948 is full of inconsistencies, many of which can be easily corrected. For example, the Council desires to foster generic names and yet often gives emphasis to trade-marked names apparently unintentionally. Generic names are in bold face capitals to distinguish them from protected names which are in bold face upper and lower case, i.e., AMOBARBITAL—Amytal—Lilly. However, in the case of substances for which generic names have not yet been adopted, the registered trade-mark name appears in bold face capitals, i.e., DIGIFOLIN—Ciba. This seems to give a slight advantage to those firms which have not complied with the Council's request.

In the preservative field, the generic versus protected names issue seems to be particularly unfairly treated at times. For example, on page 489 under Influenza Virus Vaccine it is noted that Lilly's product is preserved with "Merthiolate" while for the Squibb, Sharp and Dohme, and the Pitman-Moore products the chemical name "sodium ethyl mercuri thiosalicylate" is employed.

In a number of places it is evident that a standard style book would be of great assistance. The basic arrangement in subsections seems to be alphabetical. However, on pages 52–53, Butethamine Formate and Hydrochloride appear between Metycaine Hydrochloride and Phenacaine Hydrochloride evidently because they are alphabetized under their trade names, Monocaine Formate and Hydrochloride.

The treatment of "salts" of organic compounds is not always uniform. In the case of the sulfonamides, the sodium compounds are all placed together in a separate section following the sulfonamides themselves (pp. 144–148). In the case of the soluble barbiturates, the sodium compound follows the "acid" form, i.e., amobarbital is followed by amobarbital sodium (see pp. 448 ff.).

Dosage forms of a drug are not all treated uniformly either. For example, epinephrine, epinephrine solution, and epinephrine in oil each rates a separate monograph (pp. 233–241). In the case of penicillin, there is one basic monograph which covers all recognized penicillin dosage forms.

Frequently there is an inconsistency in the chemical nomenclature (especially noticeable in the barbiturates), which is most confusing to physicians, pharmacists, and even to organic chemists. A similar inconsistency is the alphabetizing of isomers, i.e., *meta*cresyl acetate is under *c* but *para-aminobenzoic acid* is under *p*.

A valuable change in N. N. R., actually brought about in 1947, is the separation of the tests and standards from the "physicians' part of the book." In general, Section B on tests and standards is well done and is useful. Correlating tests a little more closely with U. S. P.-N. F procedure would be very helpful to the research workers on these compendia since frequently new admissions to these official books come from N. N. R.

Because of the rapid appearance of "miracle" drugs, each year sees the book becoming a little more unwieldy. It is believed that careful examination of scope and the developmen of a consistent style can obviate many of thes difficulties. It is in this spirit that the reviewe has the temerity to call attention to these matters

The editors, the members of the Council, th members of the laboratory staff, and all who partic pated in this enterprise are to be congratulate upon doing an amazing volume of work in a ver short time and making a real contribution to th pharmaceutical and medical professions.

Trade-Mark Law and Practice. Lanham Ac Edition. By LEON H. AMDUR. Clark Board man Company, New York, 1948. xiii + 77 pages. 15 x 24 cm. Price $15.00.

For many years execution of the trade-mark law like patent law, has been a specialized and a essential operation. Since a large number of drug and drug products have trade-marked names, thi phase of law is of tremendous importance to th drug industry.

This book gives the Trade-Mark Act of 194 (the Lanham Act), the Trade-Mark Rules of th U. S. Patent Office and an interpretation of th many factors in trade-mark law by citing cases.

Descriptiveness, geographical connotations, sur names and corporate names, secondary meaning radio-marks, generic designation, similarity o goods, related companies, abandonment and man other problems relating to the trade-mark are a important to the drug field as to other fields Nearly any problem in trade-mark law in thi book cites a case in the drug field. The bool is well documented with case citations an is indexed by means of a general index, an index t patent office forms, an index to the sections o the 1946 Act referred to in text, an index to prio acts, and an index to patent and trade-mark rule referred to in text.

To anyone interested in the intricacies of trade mark law, Mr. Amdur's book will be of materia assistance.

Practice of Allergy. 2nd ed. By WARREN T VAUGHAN and J. HARVEY BLACK. C. V. Mosby Company, St. Louis, 1948. xx + 1132 pp 16 x 25 cm.

Although *Practice of Allergy* was written for the medical practitioner, some pharmacists will have occasion to consult it for reference.

The history of allergy and anaphylaxis makes fascinating reading as do the more general phases of allergy. Climate, social status and environmental factors in relation to the incidence of allergy are of general interest to all in the health field. By sharpening the pharmacists' interest in allergy, no doubt many seekers after advice on self-medication will be sent to proper physicians.

Some pharmacists, particularly those practicing in hospitals, will be interested in the detailed methods for preparing and standardizing allergens for diagnostic testing and for desensitization. Others will find interest in techniques of pollen counting and identification of pollens and other allergens (aerobiology). Of especial importance is the section of the book dealing with drug allergy.

A short chapter on treatment using antihistamin and other [illegible] is also included.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Partially Hydrolyzed Dextran*

By ALICE G. RENFREW and L. H. CRETCHER

A high-molecular weight nontoxic colloid as a substitute for plasma in intravenous injection would find certain uses. Partially hydrolyzed dextran has been considered in this field (2). The experimental work presented is a study of the results of autoclaving dextran solutions in the presence of very dilute acid as a method for achieving mild hydrolysis. Viscosity measurements are reported. Fractionation with alcohol presented the problem of two-phase formation. After preliminary animal studies, it was not deemed advisable to continue the work with a view to using dextran on patients with nephrosis.

REPEATED efforts have been directed toward the search for a nontoxic colloid of high molecular weight to serve as a substitute for plasma in intravenous injection, or as a diuretic agent in the treatment of nephrotic edemas. A recent editorial in the *Journal of the American Medical Association* (1) provides a reminder that there still exists a need for plasma substitutes. During World War II the use of partially hydrolyzed polysaccharide dextran for intravenous injection was suggested by Grönwall and Ingelman (2) and this material has received clinical trial in 1000 cases in Sweden with special application to the treatment of shock and burn (2*d*).[1]

The present investigation is a study of one method which permits mild hydrolysis of dextran, i.e., the autoclaving of solutions of dextran in the presence of very dilute acid. The extent of hydrolysis was followed by measuring the decrease in viscosity of solutions subjected to autoclaving for various time intervals with water, with 0.01 *N* and with 0.001 *N* hydrochloric acid. Two studies of the fractionation of the partially hydrolyzed dextrans show that, for uses requiring rather uniform molecular size, fractionation would be necessary. Fractionation was somewhat complicated by the formation of two phases in approximately 40% ethanol.

The above hydrolytic procedures were more drastic than normal sterilization. However, a check was made of the effect of sterilization on the neutral, relatively dilute solutions to be considered for injection. In the sterilization of 5% solutions of degraded dextrans, no measurable change in viscosities was observed.

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[1] Since the present paper was submitted, two articles, appearing in a recent issue of *The Lancet*, have reported studies of dextran as a plasma substitute (10). The production and the molecular association of dextrans have been investigated (11).

Dextran is a glucose polymer which forms as a viscous gel during the growth of certain strains of *Leuconostoc* and other soil streptococci on a sucrose medium. The polymer is made up of α-glucopyranose units linked in the 1:6 position (3, 4, 6). "Degraded dextrans" have been described by Stacey and Youd (4*a*) and by Grönwall and Ingelman (2*a*, *c*). Stacey and Youd (4*a*, 4*b*) have eliminated nitrogen by heating dextran at 100° with 0.1 *N* HCl for fifteen minutes, and have found the relative viscosity of a 1% aqueous solution of the "degraded" product to be 1.01 as compared with a relative viscosity of 3.20 for 1% solutions of the unhydrolyzed dextran. Grönwall and Ingelman (2*b*) report a nitrogen-free preparation. They graph the viscosity of a number of "partially degraded" dextrans, and for intravenous testing (2*c*, page 10), they recommend a relative viscosity between 3.5 and 7 for 4% aqueous solutions of preparations; or in a more recent paper, Ingelman (2*d*) suggests a 6% solution but does not give a numerical viscosity range.

A large part of our work was carried out with a dextran which was supplied to us by the courtesy of Refined Syrups and Sugars, Inc.;[2] micro-Kjeldahl analyses showed only 0.03% to 0.04% of nitrogen. This gel had been stored under isopropyl alcohol (*p*H = approximately 6) for some months before we isolated the dehydrated white fibrous dextran. Dextran, which was obtained in our own laboratory[3] from cultures of *Leuconostoc mesenteroides*, contained 0.4% of nitrogen. The various dextran preparations were found to be soluble in ethylene diamine (7) and in acetamide as might be expected.

EXPERIMENTAL

Partial Purification of Crude Dextran.—Two-hundred-gram lots of the crude dextran were placed in cellophane sacks and dialyzed against distilled water until the dialysate gave only a slight positive test with Benedict's solution for reducing sugars. Between 1 and 2 equal weights of water diffused into the sack. Precipitation was carried out by adding the dextran solution slowly to a gallon of 95% alcohol with mechanical stirring. The thread formed best as the solution became about 85–90% of alcohol. The sample was finally dried at 110°. The yield was approximately 35% of the weight of the crude gel and had a specific rotation of +166°. After re-precipitation and re-drying the dextran: $[\alpha] = +181°$; moisture = 8–9%.

Autoclaving.—Autoclaving as a method of hydrolysis was carried out at 15 lb. pressure and a temperature of 250° Fahrenheit with the solutions in loosely-capped, screw-top bottles. The concentration of dextran, the concentration of acid, and the time intervals were varied.

The check on possible hydrolysis during sterilization of dilute neutral solutions was carried out with a 5% aqueous solution of partially degraded dextran. The relative viscosities of the 5% solution before and after hydrolysis were 4.59 and 4.56 centipoises, respectively.

Recovery.—In routine practice the solutions of "degraded" dextrans were filtered through Johns-Manville Celite to remove a flocculent sediment and reprecipitated by slow addition of the aqueous solution to alcohol to form an 80% alcoholic supernatant. The gum was redissolved, precipitated from a large volume of alcohol, and dehydrated with acetone. In several test runs the samples showed little change on subsequent extraction with 50% alcohol or after dialysis in a cellophane sack.

Viscosities and Fractionation.—Viscosities are reported in Tables I and II. For the values reported in Table I, the autoclaved solutions were filtered with suction to remove slight flocculent precipitates and aliquots of the hydrolyzates were diluted to volume to provide the 5% and 1% solutions tested. It is evident from viscosity measurements reported in Table I that the average molecular size is appreciably decreased by autoclaving even aqueous solutions at 15 lb. pressure for 4–5 hours. In the presence of dilute acid, the degree of partial degradation of dextran during convenient intervals of autoclaving appears to be fairly reproducible and to show an expected correlation with the hydrogen ion concentration.

For a better knowledge of the range of molecular weights in the "partially-degraded" fractions, it was suggested that a sample of each be distributed into three approximately equal fractions.[4] This procedure gave evidence of a wide range of molecular weights in the "degraded" preparations. Such a procedure could provide one possible means of repeated fractionation toward colloids of limited molecular-weight range. Actually a complication developed in attempts to fractionate with varying concentrations of aqueous alcohol. On the careful addition of alcohol to dilute aqueous solutions of dextran (2% to 6%), clouding occurs and subsequently two liquid phases form. For the degraded dextrans studied, the two phases appeared when the

TABLE I.—VISCOSITIES OF AUTOCLAVED SOLUTIONS OF DEXTRAN

Concentration	Time[a]	η 1%	η 5%
Original	...	3.72	..
10% dextran in H_2O	1 hr.	3.11	..
10% dextran in H_2O	4 hr.	2.95	..
10% dextran in 0.001 *N* HCl	15 min.	2.73	..
20% dextran in 0.001 *N* HCl	1 hr.	2.34	17.9
20% dextran in 0.0032 *N* HCl	1 hr.	1.48	4.9
20% dextran in 0.01 *N* HCl	15 min.	1.53	7.2
10% dextran in 0.01 *N* HCl	15 min.	1.28	6.6

[a] 15 lb. pressure at 250° F.

[2] We take pleasure in acknowledging our indebtedness to Refined Syrups and Sugars, Inc., of Yonkers, N. Y., for the generous gift of dextran.

[3] The bacteriological work was carried out by W. W. Carlson who had previously studied dextran (5).

[4] We are indebted to Dr. R. C. Briant for suggesting this method of studying the distribution and range of molecular weights of samples.

'ABLE II.—FRACTIONATION IN AQUEOUS ALCOHOL (40% ALCOHOL)

	η_r		$[\eta]^a$	
	5%	1%	5%	1%
)egraded V	19.0	2.0	0.52	0.694
Upper layer	4.0	1.38	0.28	0.38
Gelatinous lower layer	27.0	2.3	0.659	0.833
)egraded II	2.35	1.35	0.17	0.30
Upper layer	3.18	1.29	0.230	0.255
Gelatinous lower layer	4.63	1.50	0.307	0.40

[a] $[\eta] = \frac{2.303 \log \eta_r}{\text{conc.}}$ (9).

queous-alcoholic solvent contained 35–45% of lcohol at 27°. Somogyi (8) has reported a similar olubility for glycogen.

Two samples of fractionation are presented in 'able II. Dextran V had been prepared by autolaving 35 Gm. of dextran with 175 cc. of 0.04 *N* ıydrochloric acid (i.e., a 20% dextran solution) for)0 minutes at 15 lb. pressure and 250° F. Thirtyıne grams of degraded dextran were recovered after ıecipitating three times and had a relative vis-osity of 2.0 for a 1% aqueous solution. Fractionaion in 40% alcohol gave a recovery of 66% of the legraded dextran in the lower, more viscous layer. 'he other sample in Table II was obtained by hyıolyzing dextran with 0.1 *N* hydrochloric acid as lescribed by Stacey and Youd (4*a*) and shows much ess spread in the average molecular weight of the 'ractions. It is noticeable that the apparent intrinsic ·iscosity of these preparations increases with dilu-ion.

Animal Testing.—The possible effects of various ıamples of degraded dextran were tested by intra-ıenous injection into mice and rabbits by Drs. M. L. Menten and A. M. Carpenter of the Children's Hos-ıital of Pittsburgh. The kidneys of these animals showed only minor microscopic changes. There was, however, evidence that the dextran was being taken out of circulation by liver and spleen. Because of the reaction in these two organs it was not deemed advisable to continue the work with a view to using dextran on patients with nephrosis.

SUMMARY

1. Partial hydrolysis of dextran has been studied by autoclaving solutions of various concentrations in the presence of water or dilute hydrochloric acid. Viscosities of hydrolyzates are reported.

2. Relative viscosities are reported for some recovered partially hydrolyzed dextrans.

3. Sterilization by autoclaving for thirty minutes caused no measurable change in the relative viscosities of 5% solutions of partially hydrolyzed dextrans.

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A Quantitative Assay Method for Pyrogens*

By WALTHER H. OTT

A quantitative assay is described for the determination of pyrogens in water and in biological products. Evaluation is based upon a standard curve, the derivation and application of which are explained. Individual rabbits were found to differ widely and characteristically in their response to a standard pyrogen. Animals used in the experiments reported were chosen so that variability was minimized.

IN A RECENT study of factors influencing the evaluation of pyrogen tests (1), data were presented indicating a direct relationship between the logarithm of the dose and the average maximum temperature rise following intravenous injection into rabbits. Reasonably satisfactory linearity was observed over a 500-fold range in dosage. This finding has now been employed in developing a quantitative assay method for pyrogens in water and biological products.

The method of evaluation employing a standard dosage-response curve as described in the following assay procedure is a new method for pyrogen tests and represents a distinct improvement in bioassay technique over the essentially

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"all-or-none" procedure currently used by many laboratories. The fixed standard curve is satisfactory from the biometrical standpoint as long as experimental error is not small enough to indicate significant differences between individual assays.

ASSAY PROCEDURE

All the tests were conducted under conditions of routine procedure employing the techniques reported from this laboratory (1, 2). Male albino rabbits received from various sources and weighing from 2 to 4 Kg. were used. No attempt was made to restrict normal growth of the animals. Instead, they were given free access to an adequate laboratory diet except while on test. Each animal was given a rest period of at least three days between tests.

The animals were restrained for the duration of the test in specially constructed boxes. Rectal temperatures were determined to the nearest 0.1° C. by the use of stationary rectal thermocouples. Iron and constantan wires of No. 24 B & S gauge (Leeds and Northrup Co., Inc.) were used for the thermocouples and leads which were connected directly to selector switches having low thermal potentials. Soft plastic tubing having an outside diameter of 3 mm. was employed as conduit for the wires.

Each rabbit was subjected to a training period of several weeks (two tests per week) before being used in the quantitative assays. The training period varied in length with individual rabbits and was necessary for valid responses in the initial tests with new animals. For the first week or two the animals were merely held in the boxes for the duration of a test to become accustomed to the restraining procedure. Then they were given two mock tests (no injections) per week until their control temperatures fell within the acceptable range of 37.0° to 38.9° C. for two successive tests. This temperature range, while lower than that specified in the U. S. P. XIII pyrogen assay, appears to be the normal for animals handled and treated according to this assay procedure.

Control temperatures were read after the rabbits had been held in the boxes for one and one-half to two hours and were followed immediately by the injections. Three subsequent temperatures were read at hourly intervals. The maximum temperature rise following the injection was calculated as the difference between the control temperature and the highest of the three subsequent hourly temperatures. The maximum rise was recorded as zero whenever none of the subsequent temperatures was higher than the control temperature.

In assaying water and biological materials, each sample was tested on three animals, the same dose per Kg. being given each rabbit: When greater accuracy was desired in the result, 4, and occasionally 5, animals were used per sample. Four rabbits per sample appeared to be most efficient on the basis of animals and effort required and precision obtainable. The average of the maximum rises thus obtained was used in determining the equivalent pyrogenicity by reference to a table of values (Table I) calculated from the standard curve.

For the purpose of the quantitative assay, a "Pyrogen Unit" was established as the pyrogenicity present in 0.1 mg. of a dry pyrogen standard ("PS1").[1] This material was prepared by saturating with sodium chloride a pyrogenic vaccine (a Pseudomonas) which was then precipitated with ethanol and dried with ether. The above unitage was chosen because under the conditions of the test the intravenous injection of 0.1 mg. of this material per Kg. (one pyrogen unit) produced an average thermogenic response of 1.0° C. per rabbit.

TABLE I[a]

Maximum Temp. Rise, Av. ° C.	Pyrogen Units in Dose/Kg.,	Maximum Temp. Rise Av. ° C.	Pyrogen Units in Dose/Kg.
0.0	0.05 or less	1.3	2.4
0.1	0.07	1.4	3.3
0.2	0.09	1.5	4.4
0.3	0.13	1.6	5.9
0.4	0.17	1.7	7.9
0.5	0.23	1.8	11
0 6	0.31	1.9	14
0.7	0.41	2.0	19
0.8	0.55	2.1	26
0 9	0.74	2.2	35
1.0	1.00	2.3	46
1.1	1.3	2.4	62
1.2	1.8	2.5	84

[a] Values for "Pyrogen Units in the dose per Kg." corresponding to "average maximum temperature rise in ° C." as calculated according to the standard curve: "average maximum temperature rise in ° C. = 1.00 + 0.78 (log pyrogen units in the dose per Kg.)".

In order to insure continuously uniform sensitivity of an average response of 1.0° C. to one pyrogen unit per Kg., the rabbits in the assay colony were periodically tested with the pyrogen standard. Animals that repeatedly gave responses of less than 0.6° C. or more than 1.7° C. to one pyrogen unit were removed from the colony.

The linear equation for the standard dosage-response curve as experimentally determined in this laboratory was $Y = 1.00 + 0.78X$. In this equation Y is the average maximum rise in ° C.; $1.00 = a$, which is the average maximum rise for one pyrogen unit per Kg. (that is, when $X = 0$); $0.78 = b$, which is the slope of the curve (that is, an increase of 0.78° C. in maximum rise per tenfold increase in dose of pyrogen); and X is the logarithm of the dose per Kg. in pyrogen units.

By this method of evaluation the number of pyrogen units is estimated directly for a given quantity of substance (i.e., the dose administered). Until the pyrogen, or pyrogens, is chemically defined and potency can be stated in terms of weight of a pure compound, the pyrogen unit as defined above should suffice as a measure of pyrogenic activity.

EXPERIMENTAL

The position ($a = 1.00$) and slope ($b = 0.78$) for the fixed standard curve were adopted as the result of a series of multi-dose experimental assays designed to furnish information on precision and variability of the testing procedure. For these assays, animals were selected at random from the test animals in use at the time. Two or more graded doses

[1] The author is indebted to Dr. D. M. Tennent for preparation of the pyrogenic powder used in these studies.

of the pyrogen standard were used in each assay and equal numbers of animals were given each of the doses in an assay. Each assay was performed on a different day. Biometrical analyses of the data were conducted according to methods described by Snedecor (3) and Bliss (4).

Results of these experimental assays (Table II) showed that both the sensitivity to pyrogen and the slope of the dosage-response curve were apparently maximum and relatively constant in rabbits averaging over 2 Kg. in weight and under 39° C. in control temperature (Expts. 2 and 3). In younger, smaller rabbits (Expt. 1) both sensitivity and slope were appreciably less although all animals had been presumably well trained prior to the tests. Nevertheless, variability tended to be lower in the heavier animals, which in addition had been used in routine assays for a longer time.

The pooled slope of 0.78 calculated for Expts. 2, 3, and 4 was therefore considered the most reliable estimate available for the slope of the curve with animals weighing over 2 Kg., having control temperatures below 39° C., and showing sensitivities of approximately 1° C. to one pyrogen unit per Kg. It was interesting to note that an analysis of covariance of the data presented by Robinson and Flusser (5) showed the slope of their dosage-response curve to be 0.79 (mean square for linear regression = 25; $F = 173$ *HS*). The fact that linear regression has been very highly significant whenever tested ($F = 15$ to 98 in Table II) is further proof of the existence of the dosage-response relationship in the pyrogen assay.

An inverse relation between body weight and control temperature was indicated in the experiments cited in Table II. This relationship was found to be

TABLE II.—SUMMARY OF EXPERIMENTAL ASSAYS WITH A STANDARD PYROGEN ("PS1")

Experiment		1	2	3	4	5
Mean Body Wt., Gm.		1690	2250	3110	2970	3210
Mean Control Temp., ° C.		39.15	38.78	38.60	38.06	38.40
Source of Variation[a]						
Between assays	*df*	7	1	2	2	2
	M.S.	0.426	0.547	0.032	0.262	0.138
	F	2.1 *NS*	2.8 *NS*	0.3 *NS*	2.6 *NS*	1.1 *NS*
Linear regression	*df*	1	1	1	1	1
	M.S.	11.2	12.8	10.8	7.59	1.93
	F	56 *HS*	64 *HS*	98 *HS*	77 *HS*	15 *HS*
Error	*df*	59	63	32	20	20
	M.S.	0.200	0.198	0.110	0.099	0.120
	F	1	1	1	1	1
Sensitivity, a[b]		0.80	1.11	1.04	1.20	0.57
Slope, b		0.60	0.77	0.78	0.80	0.40
Variability, s		0.447	0.445	0.332	0.315	0.347
Precision, s/b		0.75	0.58	0.42	0.39	0.86

[a] As determined in the analysis of covariance between log dose per Kg. and ° C. max. temp. response; *df* = degrees of freedom, *M.S.* = mean square, *F* = ratio between indicated variance and error variance, *HS* = highly significant source of variation ($P < 0.01$), *NS* = not significant.

[b] a and b in linear regression equation "average maximum temperature rise = $a + b$ (log pyrogen units per Kg.)." a is the average maximum response to one pyrogen unit per Kg., and b is the slope of the dosage-response curve. s = standard deviation, the square root of the error mean square in the analysis of variance.

The value s/b is one standard deviation in terms of log dose. Low values indicate high precision.

Neither differences in mean responses to pyrogen ("Between assays") nor in individual slopes (F = 1.0 or less, not tabulated) were significant. Consequently, use of the fixed standard dosage-response curve was valid because individual assay curves did not differ significantly among themselves. Should situations occur in which position or slope of the response curve or both differ significantly from one assay to another, it would be preferable to determine, and apply, the position and slope of the curve for each assay.

In a group of rabbits selected especially for high sensitivity, the sensitivity in the experimental assays (Expt. 4) was slightly above that in Expts. 2 and 3 while the slope was essentially unchanged. In a second group of rabbits, selected for low sensitivity, sensitivity and slope were so low (Expt. 5) that such animals were regarded as unsuitable for use in acceptable quantitative assays. Because the above results indicated a tendency toward higher sensitivity in animals having low control temperatures and high body weights, the minimum weight used for routine assays was increased to 2 Kg. The maximum weight has been approximately 4 Kg. largely as a matter of convenience.

very marked in the assay colony when animals weighing 1.3 to 2.9 Kg. and averaging 2.10 Kg. were used (1), as shown in Fig. 1. In the higher weight range of 2 to 4 Kg. this relation apparently ceased to exist. During a twelve-month period in which over 96% of the animals weighed between 2 and 4 Kg., the average control temperature remained uniformly close to 38.5° C. throughout this weight range.

The heavier animals in most instances had been subjected to the routine procedure for several months longer than the lighter animals, and consequently were expected to be more accustomed to the test. However, recent experience indicates that previously unused rabbits of 2 to 3 Kg. show the typically low control temperatures after relatively short training periods. Apparently the relation between control temperature and body weight (and age) is not primarily a function of training. The high weight range has resulted in increased uniformity of the control temperatures, which probably accounts for some of the decrease in variability of responses to pyrogen (Table II).

By means of the periodic tests on each animal in the assay colony with one pyrogen unit per Kg. for

the purpose of maintaining uniform responses to pyrogen, it was found that individual rabbits in general appeared to have characteristic sensitivities to pyrogen. Maximum temperature rises of the 169 rabbits in the initial test ranged from 0.0 to 2.8° C. with a mean of 1.13° C. and a standard deviation of 0.47° C. A retest with one pyrogen unit per Kg. on the 15 animals giving rises of 1.8° C. or above and on the 12 rabbits giving rises of 0.5° C. or less in the initial test showed that responses in the retest closely paralleled the responses in the first test (Table III). How long a characteristic sensitivity to pyrogen is maintained by the individual rabbit has not yet been determined. Although the rabbits with high sensitivity might be desirable from that standpoint, the proportion has been too small to be of value at present (5 to 9%).

The periodic tests with one pyrogen unit per Kg. have also shown that the distribution of the individual maximum temperature rises conforms closely to the normal distribution (Table IV). In the first test, referred to above, the distribution was slightly asymmetrical (significant $g\ 1, P = 0.02$). However, in the next two tests, eight and eleven months later, the distributions did not deviate significantly from normal. Under such conditions the mean of a group of maximum rises is expected to provide an unbiased estimate of the pyrogenic response. The mean is therefore more reliable than any individual maximum rise.

TABLE III.—COMPARISON OF RESPONSES IN TWO GROUPS OF RABBITS SELECTED ON THE BASIS OF SENSITIVITY TO PYROGEN. THE DOSE IN EACH TEST WAS ONE PYROGEN UNIT PER KG.

Relative Sensitivity	No. Rabbits	Test	Maximum Temperature Rise Mean, ° C.	Range, ° C.	Mean Control Temp., ° C.	Mean Body Weight, Kg.
High	15	1	2.02	1.8–2.8	38.12	2750
		2	1.70	1.0–2.3	38.25	2820
Low	12	1	0.33	0.0–0.5	38.72	2900
		2	0.74	0.5–1.3	38.58	3000

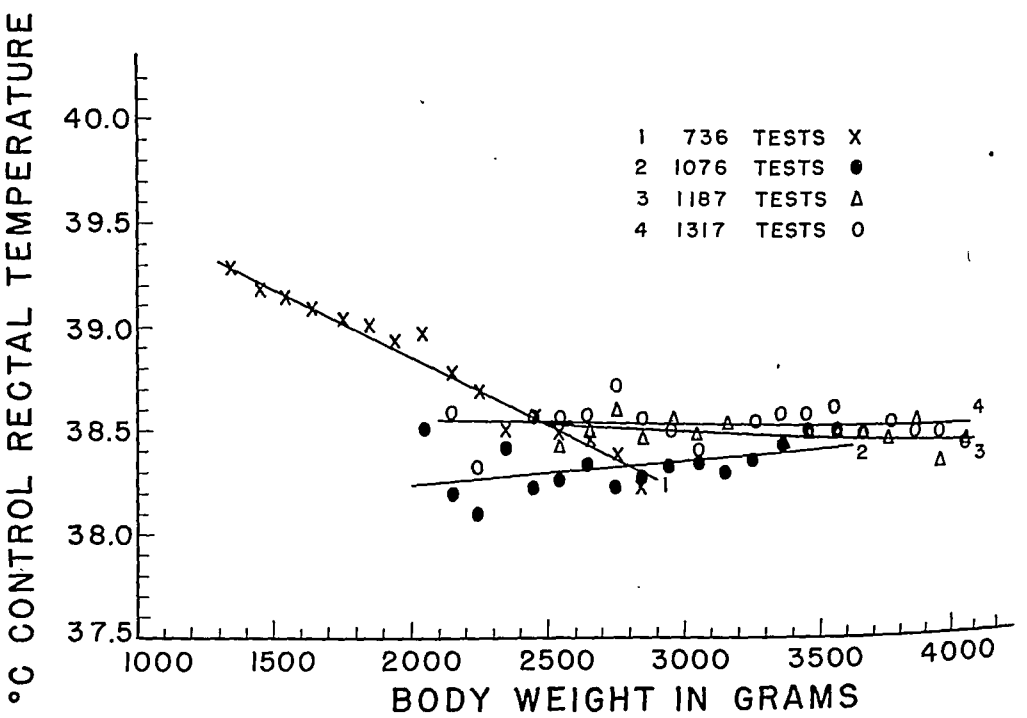

Fig. 1.—Curves showing the relation between control temperature and body weight for different weight ranges of rabbits restrained for one and one-half to two hours in special boxes for the pyrogen assay. Each point represents the average control temperature of 12 to 152 animals whose weights fell within the corresponding 100-Gm. interval during the course of one month's routine tests.

Curve I represents data obtained one month prior to institution of the quantitative assay. Curves II, III, and IV show the constancy of average control temperatures of rabbits throughout the higher weight range. These curves represent data collected during one month of routine assays 4, 8, ond 15 months, respectively, subsequent to the period represented by Curve I.

TABLE IV.—STATISTICS ON MAXIMUM TEMPERATURE RISES FOLLOWING INJECTION OF ONE PYROGEN UNIT PER KG. TO ALL RABBITS IN THE ASSAY COLONY

Test No.	No. Rabbits	Mean Rise, ° C.	Standard Deviation, ° C.	Deviations from Normality[a] $g1 \pm S.E.$	P	$g2 \pm S.E.$	P
1	169	1.13	±0.47	0.44 ± 0.19	0.02	0.62 ± 0.37	0.10
2	165	1.09	±0.42	−0.17 ± 0.19	0.37	0.03 ± 0.38	0.99
3	150	1.09	±0.39	0.24 ± 0.20	0.22	0.11 ± 0.39	0.80

[a] Calculated by procedures described by Snedecor (3).

In the previous study (1) it was found that the maximum rise was inversely proportional to the control temperature in addition to being a function of the dose of pyrogen. Some progress has been made in reducing the effect of control temperature so that the maximum rise is more closely related to the amount of pyrogen administered. In the initial experiment on 169 rabbits, correlation ($r = -0.345$) and linear regression ($b = -0.338$) between maximum temperature rise and control temperature were highly significant ($P < 0.01$). Omission of the data on the 27 rabbits of high and low sensitivities, thereby leaving a range of 0.6 to 1.7° C. in response to one pyrogen unit, reduced very significantly the correlation ($r = -0.234$) and regression ($b = -0.169$). These statistics, however, were still significant ($P = 0.01$), indicating that a greater reduction in range would have been necessary to reduce the relation to nonsignificance.

Values obtained in the second periodic test (165 rabbits) were almost identical before and after omission of the data on 28 rabbits of high and low sensitivities. However, in the third test (150 animals) the correlation ($r = 0.026$) and regression ($b = 0.025$) were almost zero and were unchanged by omission of data on 17 rabbits of high and low sensitivities. It appears that periodic elimination of rabbits giving responses above 1.7° C. and below 0.6° C. to one pyrogen unit per Kg. has resulted in an assay colony in which the maximum temperature rise is not significantly affected by the corresponding control temperature under the conditions of these tests.

DISCUSSION

The precision of this pyrogen assay is relatively low compared to other biological assays (6). However, a direct means for increasing the precision of the assay value on a given sample is to increase the number of animals used. The standard error of the pyrogenicity is inversely proportional to the square root of the number of animals used in the assay, as shown in the following formula for % *S.E.* (4):

$$\%\ S.E. = \frac{s \times 230.3}{b \times \sqrt{n}}$$

Thus, the % *S.E.* could be reduced one-half by an increase of fourfold in the number of rabbits per assay. Of course, any reduction in variability (s) whenever practical, or increase in slope (b), if possible, would also tend to increase the precision.

The data in Tables II and IV indicate that the average standard deviation (s) for the individual response of rabbits under our testing conditions probably has been close to ±0.40° C. From this value the standard error of the pyrogenicity determined from the average response by a group of 4 rabbits, for example, was calculated by means of the above formula as approximately ±60% of the estimated pyrogen unitage, i.e.,

$$\%\ S.E. = \pm\frac{0.40 \times 230.3}{0.78 \times \sqrt{4}}.$$

In a series of samples varying from 0.1 to 1.0 pyrogen units per dose per Kg., and assayed from 2 to 11 times each with 4 rabbits per assay, the average standard error of an individual assay value was calculated as ±55% (error standard deviation × 230.3). Actual reproducibility thus agreed closely with that expected theoretically on the basis of inherent precision of the assay.

As is customary in adopting new methods of evaluation, each laboratory should determine the sensitivity to a standard pyrogen and the slope of the dosage-response curve under their testing conditions. The mean sensitivity thus determined should be substituted for a in the equation, $Y = a + bX$, and the observed slope used in place of b. A table of values for pyrogen units corresponding to mean responses can then be calculated similar to Table I presented above.

The experimental determination of sensitivity and slope is not difficult, as study of appropriate biometrical references will show (e.g., 4). The following example is given to illustrate an experimental assay for this purpose:

Dose, Description	Low (L)	High (H)
Dose, Pyrogen Units/Kg.	0.2	5.0
Dose, in logs	−0.699	0.699
Response, individual maximum rises in °C.	0.4	1.5
	0.7	1.4
	0.5	1.8
	0.5	1.6
Response, total	2.1	6.3

Orthogonal coefficients (apply directly to total responses);

for sensitivity $v = +1$ $+1 = 8.4$

for slope $w = -1$ $+1 = 4.2$

Mean sensitivity to one pyrogen unit per kg. = $a = \frac{v}{N_L + N_H} = \frac{8.4}{8} = 1.05°\text{C}$

Note that mean log dose is 0, corresponding to one pyrogen unit. N = number of responses to dose indicated.

$$\text{Slope} = b = \frac{w}{iN} = \frac{4.2}{1.398 \times 4} = 0.75.$$

i = log dose interval between L and H.

Corresponding equation therefore is:

Av. Max. Rise = 1.05 + 0.75 (log pyrogen units per Kg.).

For a reliable determination of sensitivity and slope, a series of such assays should be conducted on animals chosen in the same manner as for the routine tests, and the responses totaled for the calculations. Preliminary tests might be necessary to determine

whether or not the above doses provoke the expected responses. At any rate the dose interval in a two-dose assay should be at least tenfold for satisfactory evaluation of the response to pyrogen. The doses of 0.2 and 5.0 pyrogen units per Kg. were chosen because of ease in periodically checking directly the sensitivity and slope of the standard curve, as indicated in the example.

SUMMARY

1. A quantitative assay has been developed for the determination of pyrogens in water and biological products. Evaluation is based upon a standard curve having the equation "average maximum temperature rise in °C. = 1.00 + 0.78 (log pyrogen units per Kg.)."

2. A "pyrogen unit" was arbitrarily established as the amount of pyrogen in 0.10 mg. of a standard preparation. Under conditions of this test one pyrogen unit caused an average maximum rise of 1.0° C. in rectal temperature.

3. The standard error of an assay value based on the average response of 4 rabbits was estimated at approximately ±60 per cent based on observed reproducibility as well as on inherent precision of the assay.

4. Tabular values were given for evaluation of assays conducted under conditions conforming to the statistics of the standard curve, and a procedure was illustrated for determination of sensitivity and slope of the dosage-response curve.

5. Individual rabbits appeared to differ widely and characteristically in their response to a standard pyrogen. Use was made of this fact in choosing animals so that variability of responses was minimized.

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The Preparation and Composition of Groundnut Cake, *Arachis Hypogea* (Peanut) Hydrolysate*,†,‡

By R. RAGHUNANDANA RAO and P. R. VENKATARAMAN

The preparation of peanut cake hydrolysate by use of papain is described. The composition of the hydrolytic products was determined and the results are reported.

IT HAS been widely accepted that the peanut is a fairly complete food from the standpoint of its chemical composition and ready digestibility (1). It contains an abundance of energy-producing constituents in the form of fat and carbohydrate, several vitamins of the B group (7–10), and is also a very rich source of vitamin E. The work of Johns and Jones (2–4), Brown (5), and Rose (6) have shown that arachin and conarachin, the proteins occurring in the peanut, contain significant proportions of indispensable amino-acids.

In India, peanut cake, commonly known as groundnut cake, is used largely either as fertilizer or fodder. In the production of antibiotics by microorganisms (11, 12), papain hydrolysate of groundnut cake as a medium has been used with very satisfactory results comparable with those of synthetic media. In this communication the preparation and composition of the hydrolysate are described.

EXPERIMENTAL

Preparation of Materials and Proximate Composition of the Groundnut Cake.—The peanut cake was supplied by Sri RamaKrishna Oil Mills, Bangalore, India. The cake was powdered and passed through a 60-mesh sieve. The papain used in these experiments was prepared by treating fresh latex of unripe papaya with ten volumes of dry acetone, filtering through a Buchner funnel, and drying in vacuum. In Table I the mean proximate chemical composition of the cake expressed as percentage of the dry material is presented.

Preparation and Composition of Groundnut Cake Hydrolysate.—Fifty grams of the finely powdered peanut cake was dispersed in 300 cc. of water in a

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TABLE I.—PROXIMATE COMPOSITION GROUNDNUT CAKE

Determinations	Dry Material, %
Moisture[a]	5.01
Protein[c]	51.88
Ether extractives[a]	6.82
Carbohydrate[b]	26.63
Fiber[a]	4.16
Ash[a]	5.50

[a] Methods of the Association of Official Agricultural Chemists (13).
[b] Hassid, McCready and Rosenfals (14).
[c] Micro-Kjeldahl method using the digestion mixture of Chibnall, Rees, and Williams (15).

500-cc. round-bottom flask fitted with a condenser. One gram of papain was added to the mixture which was then shaken vigorously for ten minutes. The mixture was heated in a water bath maintained at a temperature of 73–77° for five hours and was shaken frequently. At the end of the period, 20 cc. of 0.5 *N* sulfuric acid was added and the mixture was boiled for fifteen minutes under reflux and then filtered hot or centrifuged. The residue was washed twice with 75- and 50-cc. portions of hot water. The combined filtrates or centrifugates and washings were clarified as necessary, by refiltration through filter paper and were made up to a volume of 400 cc. with water containing 1 cc. of chloroform.

The hydrolysate gave positive tests with the Biuret, the Millon, and the Hopkins-Cole reagents. Cystine was present in the hydrolysate in very small quantities as indicated by its faint reaction with uric acid reagent. The mean *p*H of the hydrolysate was 5.3 and gave only a faint turbidity with 5% trichloracetic acid. Under the conditions of hydrolysis, 12.5% of the carbohydrate and 66.3% of nitrogen of the groundnut cake solubilized. The composition of the hydrolysate is summarized in Tables II and III. Recovery of added tyrosine and tryptophane to the hydrolysate was good.

TABLE II.—HYDROLYSATE COMPOSITION

Determinations	Gm. per Liter of Hydrolysate			
	Expt. No. I	Expt. No. II	Expt. No. III	Expt. No. IV
Total solids[a]	57.70	52.20	57.60	55.50
Total nitrogen[b]	6.85	6.80	6.89	6.66
Amino nitrogen[c]	1.49	1.52	1.39	1.55
Amide nitrogen[d]	0.73	0.70	0.77	0.73
Phosphotungstic acid precipitable nitrogen[e]	4.25	4.12	4.38	4.33
Tryptophane[f]	0.51	0.53	0.51	0.49
Tyrosine[f]	2.18	2.04	1.86	2.03
Starch[g]	4.14	...	4.18	...
Ash[h]	2.98	...	3.02	...

[a] 10-cc. aliquot of the hydrolysate was dried at 100° to constant weight.
[b] Micro-Kjeldahl method.
[c] Sorensen formol titration method.
[d] Amide nitrogen was determined in 2 cc. aliquots of the hydrolysate according to Damodaran (16).
[e] Basic nitrogen was determined on the phosphotungstic acid precipitate after decomposition with barium hydroxide solution (17).
[f] [illegible] were determined by the method of [illegible] after hydrolyzing 2-cc. aliquots wit[illegible] oxide in a steam bath for eight hours.
[g] Method of Hassid, McCready, and Rosenfals (14) on 25-cc. aliquots.
[h] Dry material obtained from 20-cc. aliquots of the hydrolysate was ashed at 600°.

TABLE III.—NITROGEN DETERMINATION

Form of Nitrogen	Mean Value Expressed as % Total Nitrogen
Amino nitrogen	21.09
Amide nitrogen	10.76
Phosphotungstic acid precipitable nitrogen	62.79
Tyrosine	2.23
Tryptophane	1.03
Mean complexity of peptone = 4.75	

From Table II it is evident that the ash constitutes 0.3% of the hydrolysate. The ash gave positive tests for phosphorus, potassium, iron, calcium, magnesium, and aluminum. It contained on an average of three analyses 21.35% potassium (19) 0.17% iron (20), and 3.9% phosphorus (21).

SUMMARY

1. The preparation of the groundnut cake (peanut, *Arachis hypogea*) hydrolysate with papain and its composition are described.

2. Nearly 66 per cent of the total nitrogen of the groundnut (peanut) cake could be dissolved under the conditions of the experiment.

3. The hydrolysate contains a peptone of a mean complexity of 4.57. Of the total nitrogen of the hydrolysate 63 per cent is precipitable by phosphotungstic acid. Tryptophane and tyrosine are present in significant amounts.

4. Ash constitutes 0.3 per cent of the hydrolysate and has a very high potassium content with appreciable quantities of iron and phosphorus.

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Influence of Amphetamine Sulfate on Cerebral Metabolism and Blood Flow in Man*,†

By BENEDICT E. ABREU, GRANT W. LIDDLE, ARTHUR L. BURKS, VIOLETTE SUTHERLAND, HENRY W. ELLIOTT, ALEXANDER SIMON and LESTER MARGOLIS‡

Cerebral blood flow, cerebral oxygen and glucose utilization and mean blood pressure were determined in a series of humans before and after the intravenous administration of amphetamine. Amphetamine sulfate, 20 mg., increased mean blood pressure, and reduced cerebral blood flow and oxygen uptake in the majority of subjects.

THE OBJECTIVE and subjective manifestations of central nervous system stimulation produced by ampetamine sulfate in most mammals are well recognized and adequately documented in the pharmacologic and medical literature. Numerous reports have indicated the usefulness and mechanism of action of ampetamine in stimulating the depressed mammalian central nervous system. The effects on the metabolism of brain slices have been described by Mann and Quastel, (1) but an adequate investigation of cerebral metabolism in the intact mammal has been delayed because of inadequate procedures for determining cerebral blood flow. Dumke and Schmidt (2), by means of the bubble flow meter, have shown that amphetamine decreases cerebral blood flow in the anesthetized monkey, and Schmidt, Kety, and Pennes (3) have found that it decreased cerebral metabolism and blood flow in 2 anesthetized monkeys and increased it in a third. The introduction of the Kety-Schmidt nitrous oxide technique (4) has made possible the ready investigation of the effects of drugs upon the cerebral vascular bed and upon the over-all metabolism of the intact mammalian brain. It has been emphasized by Schmidt (5) that only three easily available mammals possess a cerebral vascular circuit with a minimum of anatomic peculiarities allowing for the use of such methods. These are the rabbit, monkey, and man. Since this procedure can be readily employed in man without general anesthesia as opposed to its necessity in the rabbit or monkey, a number of investigations are now in progress attempting to elucidate the action of various drugs upon cerebral blood flow and metabolism in man. The present study is concerned with the investigation of the influence of amphetamine sulfate upon cerebral blood flow and oxygen and glucose uptake in human subjects.

EXPERIMENTAL

Nine adults of different sexes with ages varying from twenty to forty years volunteered as subjects. They were all individuals in good physical health but were voluntary patients in the Langley Porter Clinic with a number of psychiatric complaints. The Kety-Schmidt nitrous oxide technique (4) or a modification devised in this laboratory (6) was employed for the estimation of cerebral blood flow. Mean blood pressure was measured by means of a damped mercury manometer which was connected to the arterial blood sampling manifold. Blood samples were obtained from the femoral artery and one internal jugular vein by means of hypodermic needles (3 in., 19 Ga.) connected by fine bore plastic tubing to separate manifolds consisting of four 3-way metal stopcocks to which were connected sampling syringes. The system was sterilized by autoclaving prior to use and was filled with sterile heparin solution (1%) immediately prior to arterial and venipuncture. The syringes were all lubricated and sealed with a fine coating of liquid petrolatum and were also heparinized.

Oxygen and nitrous oxide were determined in the blood samples by means of the Van Slyke-Neill method (7) for the determination of oxygen and by the Kety modification (8) of the Orcutt-Waters procedure for nitrous oxide. Glucose was determined in an aliquot of the same blood samples by the Somogyi-Folin method (9, 10). Glucose determinations were done in only four experiments.

A control determination of cerebral blood flow was obtained after which the subject was allowed to breathe room air for 20 minutes. During the last 5 minutes of this period amphetamine sulfate 20 mg., made to 10 cc. volume in sterile distilled water, was administered intravenously. Cerebral blood flow and metabolism were then determined during the next five to ten minutes when the mean blood pressure appeared to be stabilized.

RESULTS AND DISCUSSION

Amphetamine sulfate, 20 mg., intravenously, raised the mean blood pressure in all of the subjects but did not consistently alter cerebral blood flow, cerebral oxygen or glucose uptake. The

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results obtained in each subject are shown in Fig. 1 expressed as a percentage change from the control. Actual values for mean blood pressure, cerebral blood flow and cerebral oxygen and glucose uptake are given in Table I.

The smallest elevation of mean blood pressure was 17 mm. Hg and the greatest 60 mm. Hg. The range of variance in per cent from the control was from −40% to +14% in cerebral blood flow; from −37% to +46% in oxygen uptake; and from −38% to +33% in glucose uptake. It is of particular interest that changes in oxygen uptake, although not always of the same magnitude, were in the same direction as those in blood flow. Changes in glucose uptake could not be correlated with either. (See Fig. 1.) In six of

TABLE I.—INFLUENCE OF AMPHETAMINE SULFATE, 20 MG., I. V., ON MEAN BLOOD PRESSURE, CEREBRAL BLOOD FLOW AND OXYGEN AND GLUCOSE UPTAKE IN MAN

Subject[a]	Mean Blood Pressure, Mm. Hg		Cerebral Blood Flow, Cc./100 Gm./Min.		Cerebral Oxygen Uptake, Cc./100 Gm./Min.		Glucose Uptake, Mg /100 Gm./Min.	
	Before Rx[a]	After Rx[b]	Before Rx	After Rx	Before Rx	After Rx	Before Rx	After Rx
1	70	95	81	52	5.6	3.5	14.0	8.65
2	85	125	70	54	4.3	3.6	...	...
3	75	110	41	51	2.6	3.8	4.7	4.85
4	90	130	78	83	3.7	4.8	6.2	8.3
5	80	115	55	43	4.0	3.1	14.5	16.8
6	93	115	50	30	4.5	3.2	...	...
7	100	120	36	31	2.7	2.3	...	...
8	100	108	70	58	4.1	3.5	...	...
9	95	102	35	40	2.7	3.0	...	...

[a] Control mean blood pressure.
[b] Mean blood pressure at end of cerebral blood flow determination following Rx.

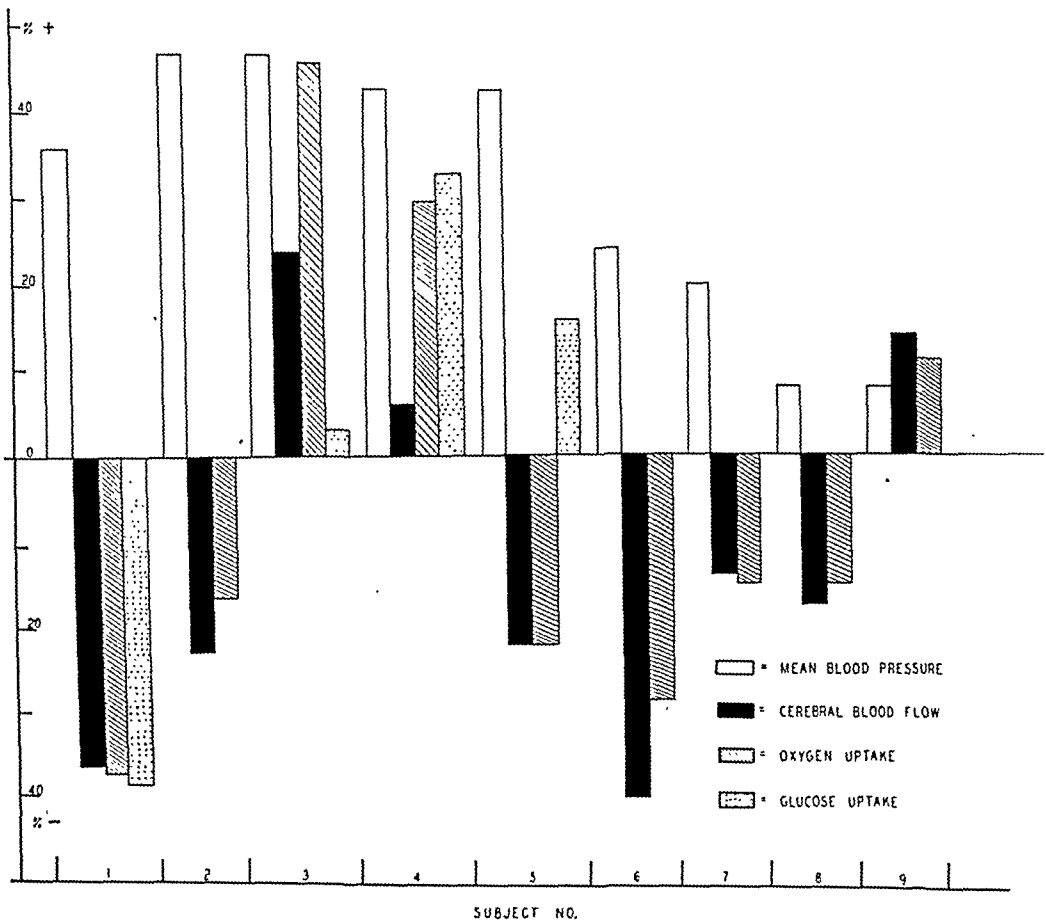

Fig. 1.—Influence of amphetamine sulfate, 20 mg., intravenously on mean blood pressure, cerebral blood flow, cerebral oxygen and glucose uptake in man (expressed in per cent from the control).

the nine subjects both cerebral blood flow and oxygen uptake were reduced. Two of the three other subjects (Nos. 3 and 4) showed a marked rise in oxygen uptake.

A correlation of these observations with the psychiatric findings and diagnosis will be published elsewhere.

From these observations it is difficult to arrive at a decision as to the effects on cerebral blood flow, cerebral oxygen and glucose uptake which should be predictable from what is otherwise known of the pharmacology of amphetamine. However, it is expected that as experience is broadened with the use of this agent in various psychic states and in "psychically normal" individuals a base line for prediction of these effects will be available.

SUMMARY

Amphetamine sulfate, 20 mg., intravenously, increased the mean blood pressure of 9 adult humans by values ranging from 17 mm. Hg to 60 mm. Hg. In 6 of the 9 subjects both cerebral blood flow and oxygen uptake were reduced. In 2 of the 3 remaining subjects cerebral oxygen uptake was definitely elevated, while in the third there was only a slight increase above this individual's control level. Glucose uptake was reduced in one, unchanged in one, and elevated in two subjects.

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Some Effects of Meperidine (Demerol) on Gastro-Enteric, Extra-Hepatic Biliary, and Cardiovascular Activity*,†

By RAYMOND W. PICKERING, BENEDICT E. ABREU, DAVID F. BOHR and WILLIAM F. REYNOLDS‡

A reinvestigation of some of the pharmacologic properties of meperidine indicates that atropine-like action cannot be demonstrated on the small intestine, extra-hepatic biliary tract or cardiovascular system of the dog. A decrease in gastric emptying in man cannot be interpreted as antispasmodic action. Evidence is cited which would place meperidine in a pharmacologic class with morphine rather than atropine with respect to its effect on structures innervated by the autonomic nervous system.

THE gastro-enteric effects of the analgetics meperidine (Demerol) and Methadone have recently been investigated by Scott and his coworkers (1) who have shown that both of these agents increase the motility of the small bowel. Gruber, Hart, and Gruber (2) observed similar effects on the duodenum and ileum of dogs. Yonkman, Noth, and Hecht (3) found that meperidine stimulated the ileum of dogs. However, it is still commonly stated that meperidine is a smooth muscle depressant and that it resembles atropine in many of its effects on other systems (3–4). In view of these apparent contradictory statements which have appeared in the literature, it was thought that a reinvestigation of some of the pharmacologic properties of meperidine on certain structures innervated by the autonomic nervous system was indicated.

EXPERIMENTAL

Animal Studies

A. **Intestinal Activity.**—The activity of the small intestine was investigated in 9 dogs anesthetized with sodium pentobarbital, 25–30 mg./Kg., intra-

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‡ We are indebted to Dr. L. L. Stanley, Chief Prison Medical Officer, California State Prison, San Quentin, Calif., who made the facilities of the prison hospital available to us. We also wish to thank Mr. W. Newcomb for his assistance in obtaining the X-ray photographs and the people who volunteered for this experiment.

peritoneally. Single balloons were placed in the jejunum and ileum through an abdominal incision and contractions were recorded on smoked kymograph paper by means of an air-water transmission system connected to sensitive Marey tambours.

B. Extra-Hepatic Biliary Activity.—A chronic preparation was utilized in which a mushroom catheter was placed in the gall bladder of a dog. The animal was trained to lie quietly without anesthesia and the activity of the gall bladder and magnitude of biliary secretion were recorded by a Trattner hydrophorograph (5) and an electrical drop recorder, respectively.

C. Cardiovascular Effects.—Carotid blood pressure was recorded by either an aneroid or mercury manometer in three dogs anesthetized with sodium pentobarbital, 25–30 mg./Kg., intraperitoneally. The responses to acetylcholine iodide, 0.01 mg./Kg., and of epinephrine, HCl 0.01 mg./Kg., intravenously were determined before and after administration of meperidine, 5 to 20 mg./Kg., intravenously.

Human Studies

Gastric Emptying.—Emptying of the human stomach was estimated by a method which essentially consisted of measuring the area of stomach which was filled with barium sulfate before and forty minutes after the intramuscular administration of 100 mg. of meperidine or 2 cc. of saline. Ten subjects received meperidine and 10 others normal saline.

RESULTS

A. Intestinal Activity.—Meperidine in doses of 0.5 and 1.0 mg./Kg. intravenously had no influence on the motility of the ileum or jejunum in 9 anesthetized dogs. When doses of 2.0, 4.0, 5.0, and 10 mg./Kg. were administered to these same animals, there was an appreciable increase in amplitude of contraction and usually some increase in rate of contraction and tone although these effects were not as marked as the increase in amplitude of contraction. The results obtained on total activity are summarized in Table I and a representative tracing is presented in Fig. 1. These results are in essential agreement with those of Gruber, Hart, and Gruber (2), Yonkman, Noth and Hecht (3), and Scott and his co-workers (1), and are quite similar to those ob-

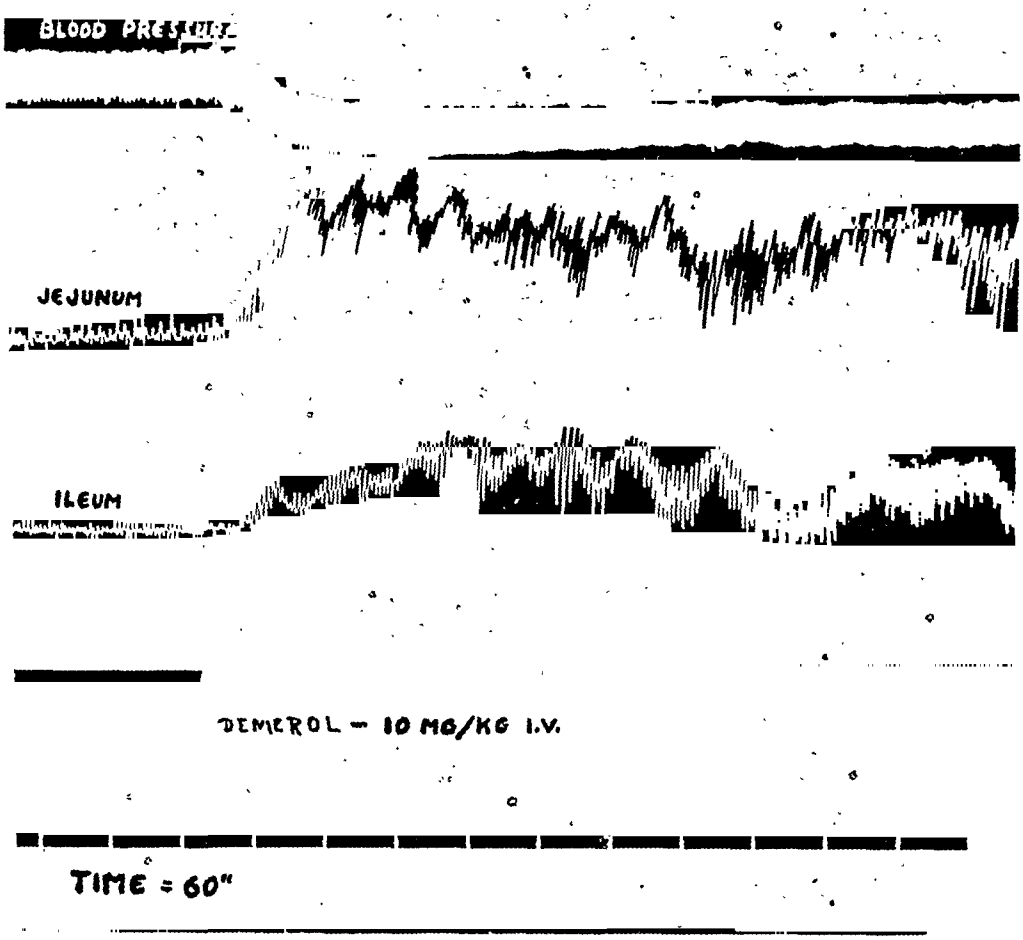


Fig. 1.—Response of small bowel to Demerol.

TABLE I.—AVERAGE RELATIVE EFFECTS OF MEPERIDINE ON INTESTINAL ACTIVITY OF THE ANESTHETIZED DOG

Dose, I. V., Mg./Kg.	Number of Observations	Amplitude of Contraction
0.5	1	0[a]
1.0	3	0
2.0	4	2+[b]
4.0	3	3+
5.0	4	3+
10.0	6	3+

[a] 0 = no change from control activity.
[b] + = increase from control activity.
Key: 2+ = moderate increase, 3+ = marked increase.

tained with morphine on the small intestine of most animals.

B. Extra-Hepatic Biliary Tract Activity.—In two experiments performed on the unanesthetized dog, meperidine, 3 mg./Kg., intravenously, increased the amplitude and number of contractions and the outflow of bile from the gall bladder. A typical record showing the effects of meperidine on the extra-hepatic biliary tract is shown in Fig. 2. These results are in keeping with the findings of Gaensler, McGowan, and Henderson (6) in man which indicated

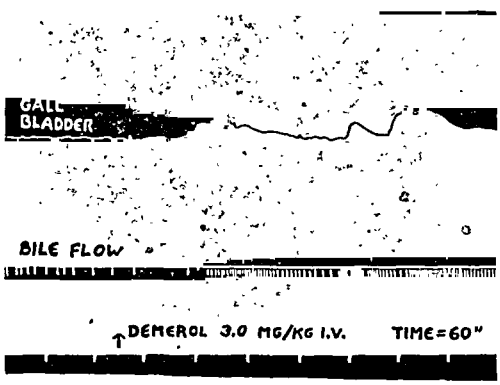


Fig. 2.—Effect of Demerol on the gall bladder.

that biliary pressure is increased by meperidine. They believe that the increase in pressure is due to duodenal spasm. In addition to this factor, it is necessary to recognize the stimulant effect on the extra-hepatic biliary tract itself.

C. Cardiovascular Effects.—Meperidine did not influence the acetylcholine-induced fall in arterial blood pressure in 3 anesthetized dogs (see Figs. 3 *A* and *B*). The response of these same animals to acetylcholine before and after the administration of a potent anticholinergic substance (β-piperidinoethyl phenyl-α-thienyl glycolate) is shown in Figs. 3 *B* and 3 *C*. Eisleb and Schaumann (7), in their pharmacologic evaluation of meperidine, stated that it possesses $^1/_{10,000}$ the activity of atropine in combating an acetylcholine-induced fall of arterial blood pressure. Meperidine itself produces vasodilation when administered intravenously and it is reasonabl to expect that this could easily account for any de crease in vasodepressor response to acetylcholin which is only $^1/_{10,000}$ that of atropine.

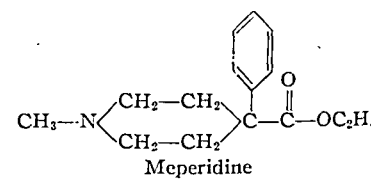
Gastric Emptying.—Meperidine reduced the emp tying time of the stomach in 10 subjects so that 55 ± 11% of the barium sulfate originally in the stomac was still present 40 minutes later. The 10 contro individuals who were subjected to exactly the sam manipulations as those receiving meperidine re tained only 24 ± 6% of the barium sulfate originall in their stomachs. A statistical evaluation of th data by Student's "t" test indicated that th probability of such an observation occurring due t chance was 1:50, thus indicating that these result are highly significant.

It may appear difficult to interpret the results o this experiment as indicating spasmogenic action o the intact human stomach; however, there are number of lines of reasoning and experimental fact which will support such a conclusion. From simila observations on the intact human stomach, it ap pears that morphine produces a sequence of event which would bring about the same results as thos found with meperidine. Morphine initially in creases gastric motility which is followed by a cessa tion of activity as shown by Abreu, Dailey, Ould Pickering and Reynolds (8). Since an agent such a tetraethylammonium bromide, a potent gastric anti spasmodic, is known to increase the size of th stomach as indicated by roentgenographic studie (9), it would be expected that if meperidine wer exerting any appreciable antispasmodic action, i would produce a similar picture. Measurements o the total area of the stomach do not support thi conclusion (8).

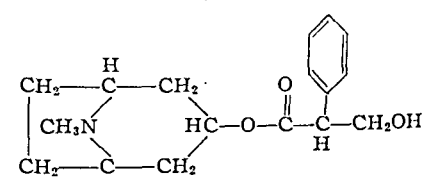
Batterman (10) has interpreted his balloo records of gastric activity as indicating an over-al depression of the stomach. By close inspection o his published records, one is led to the conclusio that meperidine produces an initial increase in gastric motility followed by a cessation of activity. In addition, his records during the period of arrested motility either show no decrease or an increase in tone. It is difficult under these circumstance to ascribe to meperidine any gastric antispasmodic action.

DISCUSSION

The structures of atropine and meperidine can be represented so that they appear similar.



Meperidine



Atropine

However, it has been shown by Blicke (11) and Lands, Nash, and Hooper (12) that atropine-like activity depends upon an amino alcohol group which meperidine does not contain. Although on casual inspection the structures may appear similar, the absence of an amino group in the alcoholic portion of the ester makes it unlikely that an atropine-like action can result from this structure. Duguid and Heathcote (13) reported a meperidine antagonism to acetylcholine depression of the isolated frog heart. However, the recent work of Scott and his coworkers (1) demonstrated that this observation did not hold for the intact mammalian heart. They have shown in trained, unanesthetized dogs that meperidine in doses of 20 mg./Kg. intraperitoneally resulted in a mean drop of cardiac rate from 72 to 48 per minute. In 2 of the animals given this dose of meperidine, tachycardia immediately resulted from the administration of atropine sulfate, 0.65 mg./Kg., intravenously. These results and the effects produced on the gastro-enteric tract are identical with those obtained with Methadone (1).

SUMMARY

1. Meperidine in doses ranging from 2.0 to 10 mg /Kg., intravenously increased the activity of the small intestine of the anesthetized dog.

2. In doses of 3 mg./Kg., intravenously, meperidine increased the motility of the gall bladder and bile flow of the unanesthetized dog.

3. There was no decrease in the blood pressure response to acetylcholine produced by meperidine in doses ranging from 5 to 20 mg./Kg., intravenously.

4. The emptying time of the intact human stomach is decreased by meperidine, 100 mg.,

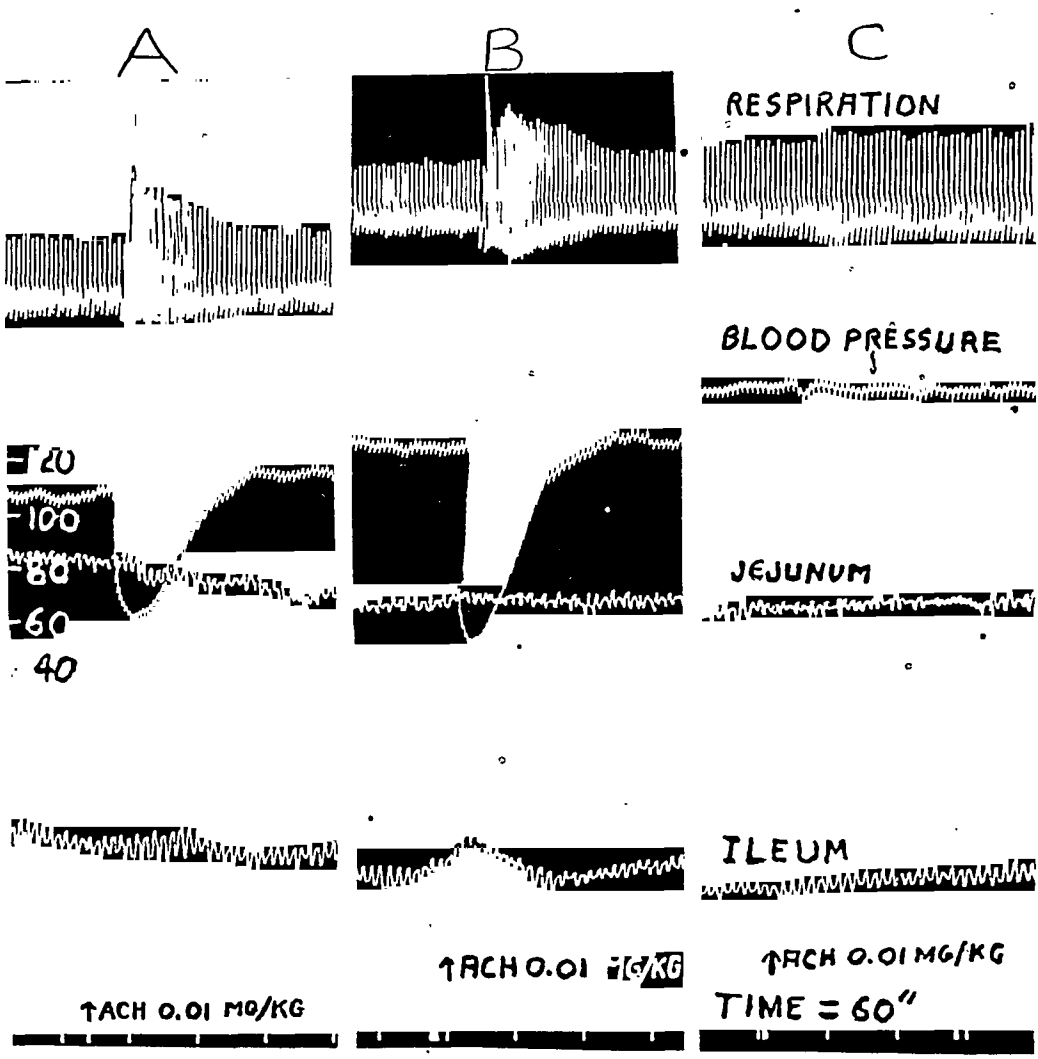


Fig. 3.—Cardiovascular effects of Demerol.

A, control response; *B*, response after 5 mg./kg. meperidine; *C*, response after ppt.

intramuscularly. A discussion of this aspect of action leads to the conclusion that this may not indicate antispasmodic activity.

5. Evidence is presented which makes it difficult to assign any atropine-like activity to meperidine.

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A Preliminary Phytochemical Investigation of the Tannin Obtained from *Pinus caribaea* Morelet*

By FRANK W. BOPE† and OLE GISVOLD‡

A new and simple technique was developed in order to remove the water soluble substances from the tannin found in *Pinus caribaea*. This tannin exhibited all the chemical and physical properties usually attributed to phlobatannins. The so-called "phlobaphene test" when applied to certain derivatives gave a red color which indicated the development of a chromophoric group whose visible absorption spectrum was in the range of that exhibited by the anthocyanins. The colored compounds could be smoothly reduced to a colorless or nearly colorless compound which in appearance was much like the original compound. Analyses of several derivatives indicated that $C_{15}H_{14}O_6$ residues might be present in the tannin molecule. The ultraviolet absorption spectrum of the original tannin and its derivatives was at about 2775 Å. which is in the region exhibited by several other naturally occurring phlobatannins and some anthocyanins.

THE LEATHER INDUSTRY is constantly consuming large quantities of both natural and synthetic tannins, and as a result the readily available sources of vegetable tannins are rapidly being depleted. This, of necessity, demands that all possible sources of future supply be investigated and utilized as far as possible.

The discovery of the presence of appreciable quantities of a natural vegetable tannin in the slash pine tree, *Pinus caribaea* Morelet, indicated the need for a study of the amount present, the physical properties, and the chemical properties of the tannin.

It is common knowledge today that one of the greatest problems involved in tannin chemistry is that of obtaining pure or homogeneous materials. Extraction of crude tannin from the bark or wood of various plants almost always results in extraction of some other plant products such as sugars, plant pigments, etc. Most tannins are amorphous substances and are exceedingly difficult to purify. The constituents of most of them are still unknown. Most tannins belong to the phlobatannin class of condensed tannins. They are related to the normal plant pigments of the benzopyran type and their nearest analogues are the flavans. The most frequent occurring nucleus of the flavan type is catechin.

According to Russell (1) phlobatannins must be built on the catechin model. Russell and coworkers (2, 3) prepared a number of synthetic compounds which they believed to be flavpinacols and they reported them to be qualitatively indistinguishable from typical natural phlobatannins. Russell, Todd and Wilson (4) measured absorption spectra of some synthetic flavpinacols and of some typical natural phlobatannins. The

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results supported the view that phlobatannins were polyhydroxy-flavpinacols.

In 1935 Freudenberg and Steinbrunn (5) stated that what Russell considered as a synthetic imitation or form of hemlock tannin (his dimer flavpinacol) doubtless possessed higher molecular weight. They regarded it as premature to assign any constitutional structure to hemlock tannin.

The object of this work was to investigate the tannin obtained from *Pinus caribaea* Morelet with the view of determining the amount of tannin present and studying its physical and chemical properties.

A preliminary pharmacological investigation of *Pinus caribaea* phlobatannin involving acute toxicity studies and the local treatment of experimental burns with a comparison to tannic acid has been carried out and will be published elsewhere.

EXPERIMENTAL

Collection and Extraction of Bark.—The inner bark or phloem of *Pinus caribaea* was collected through the courtesy of the Forest Division of the United States Department of Agriculture and was shipped from the Olustee Experimental Forest, Olustee, Fla., in the spring of 1947. The bark was air dried and ground to a No. 20 powder in a Jacobson laboratory mill.

The bark (3200 Gm.) was defatted by extraction with ether for eighteen hours in a continuous extraction-type metal percolator. The defatted bark weighed 3075 Gm. It was macerated for twenty-four hours in large evaporating dishes with 13,500 cc. of 95% alcohol. The macerated bark was packed in a large stone percolator and was allowed to stand for twenty-four hours. Percolation was then allowed to proceed slowly, more menstruum being added as needed. The total amount of 95% alcohol used to macerate and extract the defatted bark was 22,500 cc. The alcoholic extract was concentrated to dryness by vacuum distillation at a very low temperature. The yield of crude tannin was 753 Gm. or about 23% of the original dry, milled bark.

Purification.—The main task of purification was the removal of sugars. An attempt was made to purify the tannin through precipitation by means of lead acetate, but the process proved to be difficult to manipulate because of the colloidal nature of the lead-tannin precipitate and the length of time involved in washing out all the sugar.

Purification by precipitation with sodium chloride as described by Russell (1) was an improvement over the lead acetate method as far as ease of manipulation was concerned, but it proved to be very inefficient, making it necessary to find another method of removing sugars.

It was found that tetrahydrofuran would not extract sugars from aqueous solutions, but that this phlobatannin was very soluble in tetrahydrofuran and could be extracted from aqueous solution by means of a salting-out process using sodium sulfate. The crude tannin (100 Gm.) was dissolved in 800 cc. of hot water and was allowed to stand at room temperature for at least twenty-four hours, a small amount of chloroform being added as a preservative. Some red amorphous insoluble materials (4.3 Gm.) deposited and were removed by filtration. The filtrate was partially reduced or decolorized by shaking with sodium hydrosulfite, and was then extracted with 400 cc. of tetrahydrofuran in a separatory funnel. To this mixture was added an excess of sodium sulfate which effected a salting out, the tannin and tetrahydrofuran separating as the upper layer and the water, salt, and sugars as the lower layer. The aqueous layer was removed, more water was added and the salting out process was repeated. This process was continued until the aqueous layers failed to give positive tests for sugars with Molisch Reagent. The tetrahydrofuran was then removed by vacuum distillation and the resulting purified tannin weighed 42.6 Gm. or 42.6% of the original crude tannin.

Identification of Sugar.—The sugar present in the crude tannin was identified as glucose through formation of its osazone. Microscopic comparison of the crystals with those of glucosazone, rate of formation, and melting point indicated the sugar osazone was identical with glucosazone.

The sugar present gave a positive test with Fehlings solution and a negative Seliwanoff test (6) which indicated the absence of ketoses.

Properties of the Tannin.—The original crude tannin was a light red amorphous powder soluble in water, methyl alcohol, and ethyl alcohol; and insoluble in ether, benzene, and petroleum ether. It darkened in alkali and upon exposure to air it absorbed moisture. It was polymerized by heat.

The purified tannin was a slightly darker red amorphous powder with properties similar to those of the crude tannin except that it was slightly less soluble in water. It had no melting point but gradually decomposed above 195°. Its behavior when boiled with dilute mineral acids was characteristic of typical phlobatannins; it produced a bright red solution and a red precipitate of insoluble phlobaphenes.

The purified tannin was subjected to a number of qualitative tests as outlined by Russell (1). With ferric chloride it produced a dark green color; with gelatin a white precipitate was produced; lead acetate produced a light precipitate which was soluble in acetic acid; with boiling dilute HCl it produced a red solution and a red precipitate; with bromine water it gave an immediate heavy yellow precipitate; and with potassium dichromate it gave a brown precipitate.

Attempts to reduce the purified tannin with platinum black or with palladium on charcoal under 40 lb. pressure of hydrogen were unsuccessful. No apparent reduction was obtained. By refluxing 1 Gm. of tannin with 5 Gm. of zinc dust and 1 cc. of glacial acetic acid in alcoholic solution for ten minutes some decolorization or reduction occurred. This decolorization was not permanent, however, and attempts to reduce or decolorize larger amounts were unsuccessful.

Methylation with Diazomethane.—The first attempted methylation with diazomethane involved a partially reduced tannin. Ten grams of purified tannin were dissolved in 150 cc. of methyl alcohol

and the solution was partially reduced by the addition of sodium hydrosulfite. It was then methylated by diazomethane prepared from 30 Gm. of N-nitrosomethylurea in the usual manner. When an excess of diazomethane remained overnight, a light-colored precipitate was obtained that was removed by filtration, washed, and dried. The yield was about 9 Gm. of an amorphous methyl ether that had no melting point but darkened or decomposed slowly above 220°.

Better methylated products were obtained when no attempts were made to first reduce the tannin. Five grams of purified tannin was dissolved in 200 cc. of anhydrous methyl alcohol and the solution was methylated with an ethereal solution of diazomethane prepared in the usual manner. It required the diazomethane from 65 Gm. of N-nitrosomethylurea to maintain an excess of the methylating agent for twenty-four hours. The resulting pale yellow amorphous precipitate was filtered out, washed with cold water and dried in a vacuum desiccator over NaOH and then for six hours over P_2O_5 in the Abderhalden drying pistol. The yield was 3.2 Gm. of methylated tannin which decomposed at 228–230° without melting. The yield was increased to 4.7 Gm. by dilution of the filtrate with 300 cc. of cold water. Attempts to purify or crystallize the methylated tannin were of no practical value.

Anal.—Calcd. for $C_{30}H_{20}O_6(OCH_3)_6$: C, 65.24; H, 5 78. Found: C, 62.38, 62.70; H, 5.68, 5.88

Methoxyl.—Calcd.: 28.10. Found: 28.12, 28.19.

The methylated tannin produced a cherry-red solution when boiled for a few minutes with 4 drops of concentrated HCl in 10 cc. of alcohol (a positive phlobaphene test), and it produced no color with ferric chloride test solution.

Methylation with Dimethyl Sulfate and Alkali.—Because of the instability of the phlobatannin to alkali, it was necessary to methylate the tannin with dimethylsulfate and alkali in an atmosphere of nitrogen. Two grams of purified tannin was dissolved in 100 cc. of water and the resulting red-colored solution was reduced by addition of zinc dust and a few drops of concentrated HCl. The zinc dust was removed by filtration and the solution was placed in a three-necked flask equipped with a mechanical stirrer, a separatory funnel, and a tube leading from a nitrogen tank. In an atmosphere of nitrogen the solution was made alkaline by the addition of 5 cc. of 50% KOH. Some precipitate resulted and then 5 cc. of dimethyl sulfate were added at once. The mixture was allowed to become acid and then was made alkaline slowly by the dropwise addition of 5 cc. of 50% KOH. It was made alternately acid and alkaline in the same manner until a total of 35 cc. of dimethyl sulfate had been added. The total amount of 50% KOH used was 45 cc., leaving the reaction mixture strongly alkaline at the end. The total time for the addition of the reagents was about four hours and the temperature was not allowed to exceed 60°.

The methylated product was removed by filtration and when washed and dried it weighed 1.7 Gm. It did not melt but gradually darkened above 200°.

In order to obtain complete methylation, the product was remethylated in an alcoholic medium instead of an aqueous medium. A total of 20 cc. of dimethyl sulfate and 25 cc. of 50% KOH was added in small portions as previously described. The final methylated product after one precipitation from alcohol still gave no melting point but decomposed above 200° as before. It gave a negative ferric chloride test, but a positive phlobaphene test when boiled for twelve minutes. The methoxyl content was raised to 33.56% and 33.59% methoxyl by this method of methylation.

Methylation with Methyl Iodide and Silver Oxide.—Methyl iodide catalyzed by moist silver oxide was employed on the tannin previously methylated by diazomethane in an attempt to methylate a possible sterically hindered group. The procedure was similar to that described by Purdie (7). The product obtained was a light red amorphous compound which still had no melting point but decomposed above 160°. Methoxyl determinations showed 29.24% and 29.23% methoxyl indicating very little, if any, additional methylation.

Acetylation.—Acetylation by means of pyridine and acetic anhydride produced slightly pink amorphous products which did not melt but decomposed at about the same temperature as the original tannin.

Reductive Acetylation.—Acetylation with acetic anhydride and sodium acetate in the presence of zinc dust produced almost colorless acetates. The purified tannin (4 Gm.) was dissolved in 90 cc. of acetic anhydride by heating under a reflux condenser. To the resulting red solution were added 5 Gm. of zinc dust and 1 cc. of glacial acetic acid and the mixture was refluxed for ten minutes, producing a light green-yellow clear solution. The addition of glacial acetic acid and zinc dust was repeated and the mixture was refluxed another ten minutes. A third addition of these reagents was followed by 0.5 Gm. of fused sodium acetate and the mixture was refluxed for forty minutes. At the end of this time the mixture was filtered hot and the filtrate was poured slowly with stirring into 400 cc. of ice water. After standing overnight the almost colorless amorphous acetylated tannin was removed by filtration, washed thoroughly with water, and dried in the usual manner. The yield was 5 Gm. and the compound had a wide melting-point range of 103–130°. Several purifications from alcohol did not alter the melting point although an insoluble fraction amounted to 0.7 Gm. and decomposed above 170° without melting.

In order to minimize polymerization, condensation, or dehydration an acetylation was carried out at 50–55°. The procedure was similar to that just described except that the reaction was kept at 50–55° for 11 hours and then at room temperature overnight. Two grams of purified tannin yielded 2.1 Gm. of acetylated tannin which melted at 108–130°.

The acetylated tannin gave a negative test with ferric chloride and no phlobaphene test when boiled for fifteen minutes with dilute mineral acids. The addition of more acid and prolonged boiling produced a pink color indicating phlobaphene formation.

Anal.—Calcd. for $C_{30}H_{15}O_4(OAc)_6$: C, 60.39; H, 4.59. Found: C, 59.45, 59.25; H, 5.12, 5.01.

Acetyl Value.—Calcd. for $C_{30}H_{18}O_4(OAc)_6$: 37.64. Found: 37.49, 37.88.

Methoxyl determinations were carried out on the acetylated tannin to determine if any methoxyl groups were originally present in the tannin. The results showed 1.53% and 1.80% methoxyl.

Saponification of the Acetate.—About 0.3 Gm. of acetylated tannin was dissolved in 15 cc. of alcohol

with the aid of heat. In an atmosphere of nitrogen, 20 cc. of 2.5% alcoholic KOH were added drop by drop with frequent shaking. After twenty-four hours in an atmosphere of nitrogen the solution was made slightly acid with dilute HCl and 15 cc. of water were added. The alcohol was then removed by vacuum distillation leaving an amber-red solution. The tannin recovered in this manner by saponification gave a dark green color and green precipitate with ferric chloride, a white precipitate with gelatin and a positive phlobaphene test.

Fractionation of the Acetylated Tannin.—One gram of acetylated tannin was extracted with 50 cc. of ether for one hour under a reflux condenser. A second extraction was then carried out in the same manner. Evaporation of the ether solutions yielded a few milligrams of residue which, when dried, melted at 50–60°. Its properties were similar to the original acetate, and when saponified in an atmosphere of nitrogen as described, it gave positive tests with ferric chloride, gelatin solution, and boiling dilute HCl (phlobaphene reaction).

Reductive Acetylation of the Methylated Tannin.—A reductive acetylation of the tannin previously methylated with diazomethane was carried out in the usual manner. The acetylated methyl ether did not melt but gradually decomposed above 235°. It gave a negative test with ferric chloride, not producing a positive phlobaphene color until boiled over twenty minutes with dilute HCl.

Acetyl Value.—Calcd. for $C_{30}H_{18}O_4(OCH_3)_6(OAC)_2$: 11.51%. Found: 16.36%.

Lead Tetraacetate Oxidation.—If Russell's flavpinacol structure for phlobatannins was correct, it should be possible to cleave the phlobatannin molecule with lead tetraacetate. A total of 14 separate reactions were carried out using a 0.05 molar solution of lead tetraacetate in glacial acetic acid.

A carefully weighed amount (about 0.45 Gm.) of acetylated (or, in two cases, methylated) tannin was dissolved in 25 cc. of glacial acetic acid in a 250-cc. flask. To this solution was added 30 cc. of 0.05 molar lead tetraacetate and in some cases 5 cc. of methyl alcohol, in others 5 cc. of water. The mixture was kept at 50–55° for nine hours and then at room temperature for fifteen hours. The resulting reaction mixture was then titrated according to the procedure of Criegee (8) using sodium thiosulfate. The amount of lead tetraacetate consumed was 1.14 moles per mole of acetylated tannin used.

Many attempts were made to isolate cleavage products from all the reactions but none could be isolated other than recovery of what apparently was the original acetate.

Phlobaphene Study.—Purified tannin when dissolved in 10 cc. of alcohol and boiled with 4 drops of concentrated HCl, produced a bright red solution. This solution was decolorized by zinc dust, and when the red solution was shaken with sodium bicarbonate it darkened to a purple-red color. Sulfuric acid produced the phlobaphene color as well as HCl.

When the above test was carried out in water, a red insoluble material deposited. This red phlobaphene was slowly soluble in alcohol to produce a cherry-red solution which was completely decolorized by zinc dust. The decolorized solution was diluted with water and the alcohol was removed by vacuum distillation to produce a small amount of red insoluble material again.

An alcoholic solution of the tannin methylated by diazomethane, when boiled with 4 drops of HCl, produced a positive phlobaphene color in four minutes. The red color was completely decolorized by zinc dust and after removal of the zinc, heating with one drop of HCl restored the red color. The original red solution when shaken with sodium bicarbonate changed to a light purple color, and with excess bicarbonate it completely decolorized. Sodium hydrosulfite also decolorized the red solution.

The tannin methylated with dimethyl sulfate and alkali produced the red phlobaphene color much slower (twelve minutes).

The acetylated tannin when dissolved in alcohol and boiled with 4 drops of HCl produced no red color in fifteen minutes. The addition of more acid and prolonged heating produced a pink color which was decolorized by zinc dust and was restored after removal of the zinc by heating. The decolorization by zinc dust was not permanent, but the color slowly returned. Other reactions of the phlobaphene of the acetate were similar to those described for the methyl ether above.

Absorption Spectra.—Solutions containing 50 mg. of the desired tannin compound in 50 cc. of 95% alcohol were diluted ten times for use in measuring absorption spectra. The tannin compounds used were: purified tannin, reduced purified tannin, acetylated tannin, tannin methylated by diazomethane, tannin methylated by dimethyl sulfate, and the phlobaphened solutions of both methylated tannins. The solutions were measured in the Beckman Model DU Photoelectric quartz spectrophotometer using the ultraviolet sensitive phototube. In the ultraviolet region all the compounds showed maximum absorption at 2775 Å. Readings in the visible region of the spectrum were made on the Coleman Electric Spectrophotometer. There was no absorption in the visible region for any of the compounds except the phlobaphened methyl ethers. Both of the latter showed maximum absorption at 5350 Å.

SUMMARY AND CONCLUSIONS

A natural phlobatannin has been extracted from the inner bark of the slash pine tree, *Pinus caribaea* Morelet.

A new method for purification of the crude tannin involving the use of tetrahydrofuran has been presented. The sugar component of the crude tannin extract was identified as glucose.

The properties of the phlobatannin have been determined. It is an amorphous tannin. A number of reactions have indicated that it is qualitatively indistinguishable from other typical natural phlobatannins.

The phlobatannin has been methylated with diazomethane to produce an almost colorless amorphous methyl ether. Methoxyl determinations have indicated three hydroxyl groups per $C_{15}H_{14}O_6$ residue capable of methylation with diazomethane. The methoxyl content was increased by the use of dimethyl sulfate and alkali, indicating the probability of four hydroxyl groups per $C_{15}H_{14}O_6$ type residue capable of methylation.

Reductive acetylation of the phlobatannin produced an almost colorless amorphous acetate. Analytical results showed four hydroxyl groups capable of acetylation per $C_{15}H_{14}O_6$ residue or eight on the basis of Russell's suggested flavpinacol type structure.

The acetylated tannin has been saponified to yield the original tannin. Fractionation by extraction with ether produced two different melting acetates.

A reductive acetylation of the tannin previously methylated by diazomethane succeeded in acetylating an additional hydroxyl group.

Attempted cleavage of certain derivatives of the phlobatannin from *Pinus caribaea* Morelet with lead tetraacetate indicated that a simple flavpinacol structure was probably absent, although quantitative amounts of lead tetraacetate were consumed.

A study has been made of the formation of phlobaphenes. It has been demonstrated that certain substituted hydroxyl groups influence the rate of formation of the phlobaphene, due possibl to steric hindrance, but also that free hydroxy groups are not essential for the reaction. It i therefore, fair to conclude that the splitting out c water is not the primary step in the productio of the phlobaphene. It may result secondarily

Absorption spectra measurements were ver similar to those of certain well-known anthc cyanins. The anthocyanin structure may b basic or fundamental in the production of the re color in the phlobaphene reaction.

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A Phytochemical Investigation of the Kelps *Cymathaere Triplicata, Hedophyllum Sessile,* and *Egregia Menziesii**

By ROB S. McCUTCHEON,† LOUIS ARRIGONI‡ and LOUIS FISCHER§

The phytochemical investigation of three northwest marine algae is presented including a survey of the plants for their vitamin content and antibiotic properties. The results obtained are presented with a summary indicating possible usefulness of the plants.

SEA WEEDS and their products appeared as food as early as the 17th century in the history of the Japanese. For many years, agar, a gelatinous substance obtained from *Gelidium, Gracilaria*, and other genera of *Rhodophyta*, has been a common food for both the Japanese and Chinese, and it was from them that its use spread to the East Indies and later to Europe. The French used it for food in the 18th century and called it "mousse de chine." The use of agar in culture media, in medicine as a laxative, and its application in the manufacture of certain pharmaceutical preparations are well known. The Japanese also cultivate porphyra, another of the red algae, on their tidelands (1), the annual harvest being worth several million dollars. The plants are dried and flaked or powdered for the market forming a food product called Kombu. The kelps of the United States, mostly members of the brown algae or *Phaeophyta*, have found application in medicine as valuable sources of minerals and vitamins.

The red and brown algae are the important sources of commercially valuable or potentially valuable substances such as agar, carrageenin, funorin, and algin. These are all mucilaginous substances of a colloidal nature and are generally spoken of as phycocolloids. Algin is used as a food thickener, a plastic, a sizing material, an

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emulsifier, a water softener, and an excipient in pharmacy.

Of the kelps under consideration, *Cymathaere triplicata*, *Hedophyllum sessile*, and *Egregia Menziesii*, all are members of the brown algae and none has been previously studied chemically with the exception of *Egregia Menziesii*, the chemistry of the entire plant of this species having been investigated by Hoagland (2).

This investigation was undertaken to ascertain the proximate constituents, the vitamin concentration, and the mineral content of the ash, for the purpose of determining the possible value of these plants as fertilizers or food supplements. A study was made of the content of algin, laminarin, and mannitol because of their potential importance, and the fact that little is known concerning the quantities of these substances in the plants under consideration. A preliminary survey was made on the plants to determine possibilities of the presence of any antibiotic substances since they are of great importance medicinally.

EXPERIMENTAL

Collection and Preparation of the Material.—The samples used in this study were collected in late August, 1946, at False Bay near Friday Harbor, Wash. After identification by Dr. George B. Rigg of the Botany Department, University of Washington, the plants were transferred to the drying chambers in the greenhouse of the College of Pharmacy Drug Garden and were hung on wires in such a manner that there was free passage of air between the plants. The air, at a uniform 27° temperature, was kept circulating by fans in the drying chambers. A few of the plants were dried in the greenhouse proper under the same conditions except that the temperature was maintained at 27° only during the daytime. The plants were turned occasionally to prevent decomposition from contact and to promote proper drying. After the plants were dried they were ground to a No. 60 powder and stored in glass jars sealed with rubber-seated screw tops.

Two other collections of the plants were made to obtain fresh material at two different seasons for the vitamin assays. The collections, one in June and the other in September, 1947, were made in the same locality. The two collections of fresh plants were stored at 0° in a freezing unit at Friday Harbor, Wash., within two hours after gathering. After freezing for twenty-four hours they were packed in ice, properly insulated, and moved to Seattle where they were placed in a freezing unit at 0°. The vitamin assays were made on this material within six months after collection. Preceding the vitamin analyses on the fresh frozen plants, they were warmed to room temperature, dried as thoroughly as possible, and well minced before weighing. No attempt was made to separate any of the plant parts, therefore the analyses represent the amounts in the entire plant. All determinations were made in duplicate except the vitamin assays which were made in triplicate, and were repeated when necessary until checks were obtained.

Partial Proximate Analysis.—In the determinations that follow methods as outlined in the A. O. A. C. were used in every case and the results, calculated on a moisture-free basis, are presented in Tables I and II.

TABLE I.—PARTIAL PROXIMATE ANALYSIS OF THE KELPS

	Hedophyllum sessile, %	*Egregia Menziesii*, %	*Cymathaere triplicata*, %
Moisture (dried plants)	8.84	8.37	7.12
Moisture (fresh plants)	54.86	74.80	69.81
Crude fiber	3.54	10.30	6.10
Organic and ammoniacal nitrogen	2.71	2.57	1.68
Starch	Negative	Negative	Negative
Reducing sugars	Negative	Negative	Negative
Sucrose	Trace	Trace	Trace
Ash	35.27	36.25	27.97

TABLE II.—COMPOSITION OF THE ASH OF THE KELPS

	Hedophyllum sessile, %	*Egregia Menziesii*, %	*Cymathaere triplicata*, %
Water-soluble ash	27.71	23.46	23.78
Water-insoluble ash	7.56	12.79	4.19
Acid-insoluble ash	2.01	6.88	0.11
Sulfates	11.24	6.14	11.06
Calcium oxide	4.46	3.35	3.72
Magnesium oxide	4.34	1.99	3.33
Phosphates as P_2O_5	2.52	5.05	5.60
Potassium	10.43	9.40	9.86
Sodium	13.57	11.39	10.51
Chlorine	32.59	27.62	31.44
Bromine	Trace	Trace	Trace
Iodine	0.09	0.06	0.09
Alkalinity of water-soluble ash (cc. 1 *N* HCl/100 Gm.)	4.53 cc.	2.99 cc.	7.56 cc.

Extractions.—Samples of the dried plant were extracted selectively in a Soxhlet apparatus by the continuous extraction method using petroleum ether, ether, chloroform, and ethyl acetate as solvents in the order named. The extracts were evaporated, dried to constant weight at 100°, and the results are presented in Table III.

TABLE III.—RESULTS OF SELECTIVE EXTRACTION OF THE KELPS

Kelp	Petroleum Ether Extract, %	Ether Extract, %	Chloroform Extract, %	Ethyl Acetate Extract, %
Hedophyllum sessile	1.25	0.71	1.30	1.01
Egregia Menziesii	0.74	0.39	1.00	0.34
Cymathaere triplicata	3.39	0.51	0.64	0.44

Alginic Acid.—In a study on a number of species of kelp, Dillon and McGuiness (3) found a seasonal variation of algin content, the percentage being high in early spring and low in September.

The usual method for the preparation of algin is to treat the kelp with a solution of sodium carbonate; this forms the soluble sodium alginate which can be separated from the plants by filtration. The filtrate is then treated with a strong mineral acid and the liberated, insoluble alginic acid is collected on a filter.

In this study 50-Gm. samples of the dried ground plant were mixed with 4 L. of water and 50 Gm. of sodium carbonate. This mixture was heated to 80° for fifteen minutes, diluted to 12 L., and filtered. Forty cubic centimeters of 30% calcium chloride solution per liter of filtrate was placed in a container and the mixture was added with constant stirring. The resulting precipitate was collected, made up to about 12 L. with water, and the fibers broken up as much as possible. Hydrochloric acid was then added until the *p*H became stabilized at 1.4. After standing twenty minutes, the solid materials were screened off, hydrochloric acid and water were again added to bring the *p*H to 1.6, and the mixture was again allowed to stand for twenty minutes. This process was repeated, and the material was screened and washed with water until the wash water had a *p*H of 2.3 to 2.5. Concentrated ammonium hydroxide was added, mixed well, and the paste was spread out to dry.

	Ammonium Alginate, %
Hedophyllum sessile	15.79
Egregia Menziesii	13.09
Cymathaere triplicata	12 47

Laminarin.—A modified method combining some features of the methods of Barry (4), and of Colin and Ricard (5) was used for the separation of laminarin. In a study by Dillon and McGuiness (3) on the content of laminarin in kelp, it was found that the variations are substantially opposite to those of the algin content. The concentration of laminarin was found to be highest in the winter and lowest in spring and summer.

Approximately 300-Gm. samples of the fresh plant were used from the June collection. These were covered with 5% hydrochloric acid, allowed to stand forty-eight hours, filtered, and the process repeated a second time. The combined precipitates were taken up with hot water and the solution was treated with barium hydroxide in sufficient amount to give a 2% solution. This mixture was filtered and ethanol was added to the filtrate until the alcoholic content was 75%. Upon standing twenty-four hours, a flocculant precipitate of laminarin was obtained, which was separated and collected by centrifuging. The material obtained was identified by hydrolyzing with 5% hydrochloric acid and preparation of the glucosazone, which was identified microscopically and by its melting point, 205°.

	Laminarin, %
Hedophyllum sessile	0.37
Egregia Menziesii	1.66
Cymathaere triplicata	0.90

Mannitol.—Five-hundred-Gm. samples of the dried, ground plant were extracted for twelve hours with petroleum ether. The extraction was continued with 95% alcohol from which a large yield of crystals formed on standing overnight. These were filtered off, washed with boiling ether, then with alcohol, recrystallized from boiling alcohol, washed again with alcohol, and the crystals were dried in a desiccator. The melting point of the crystals was 166–168° which is identical with that of mannitol.

	Mannitol, %
Hedophyllum sessile	0.50
Egregia Menziesii	0.50
Cymathaere triplicata	0.50

Vitamin Determinations.—As previously stated all vitamin determinations were made on samples of the dried and fresh frozen plants collected at two different seasons. The results of all determinations are shown in Table IV.

TABLE IV.—VITAMIN CONTENT OF THE KELPS

Vitamin	*Hedophyllum sessile*	*Egregia Menziesii*	*Cymathaere triplicata*
Thiamine, mg./Gm.			
Spring collection, fresh	1.23	0.60	0.24
Fall collection, fresh	2.18	0.40	0.63
Dried plant	2.65	0.25	0.50
Riboflavin, γ/Gm.			
Spring collection, fresh	8.71	15.92	5.36
Fall collection, fresh	6.68	6.88	7.16
Dried plant	8.55	4.36	2.77
Niacin, γ/Gm.			
Spring collection, fresh	47.19	52.14	39.41
Fall collection, fresh	35.00	45.37	28.81
Dried plant	69.42	83.56	60.29
Pantothenic Acid, γ/Gm.			
Spring collection, fresh	5.54	40.60	10.59
Fall collection, fresh	3.02	4.54	5.91
Dried plant	54.59	50.74	48.46
Carotene, γ/Gm.			
Spring collection, fresh	8.86	15.92	9.94
Fall collection, fresh	19.05	23.89	15.24
Dried plant	6.85	2.18	3.41
Ascorbic Acid, γ/Gm.			
Spring collection, fresh	26.0	49.0	54.0
Fall collection, fresh	33.0	42.0	60.0
Dried plant	26.0	60.0	133.0

Thiamine.—The thiamine determinations were made by the thiochrome method, A. O. A. C. (6), and the results were calculated to mg. per Gm. on a moisture-free basis.

Riboflavin.—The A. O. A. C. (6) microbiological method was used. The riboflavin was extracted from the samples in the prescribed manner and a series of standard solutions was set up along with triplicate series of the unknown. Basal medium stock solution was added to each tube of the series,

brought to the same volume, sterilized, inoculated with a suspension of *Lactobacillus casei*, and incubated at 37° for seventy-two hours. The contents of each tube were then titrated with 0.1 *N* sodium hydroxide solution, using bromthymol blue as the indicator.

A standard curve was prepared from the riboflavin standard titration by plotting the average of the titration values expressed in cc. of 0.1 *N* sodium hydroxide solution, for each level of the riboflavin standard used, against micrograms of riboflavin contained in the respective tubes. The riboflavin content of the unknown solution in each tube was obtained from this curve, and the riboflavin content in each cc. of the test material for each set of tubes was calculated. The riboflavin content of the test material was calculated from the average of the values obtained from not less than three sets of these tubes which did not vary by more than 10% from the average.

Niacin.—The A. O. A. C. (6) microbiological method was used, a procedure which is similar to that for riboflavin, except that the inoculum consists of *Lactobacillus arabinosus*. The nicotinic acid content in the test material was calculated from the average titration values obtained from not less than 6 of the tubes which did not vary by more than 10% from the average.

Pantothenic Acid.—The microbiological method (7), using *Lactobacillus arabinosus*, was employed. The procedure is essentially the same as that used in the two previous assays. The pantothenic acid content of the test material was calculated from the average of the values obtained from not less than 4 tubes whose deviation from each other was not more than 10%.

Carotene.—The A. O. A. C. (6) method was used in which the sample was refluxed with alcoholic potassium hydroxide and the carotene extracted with petroleum ether. The impurities were adsorbed on magnesium carbonate in an adsorption tower and the carotene was determined in the photoelectric colorimeter against known concentrations of purified carotene. Colorimeter readings were plotted against concentrations of carotene and the curve was used for calculating the amount of carotene in the sample.

Ascorbic Acid.—The A. O. A. C. (6) method was employed in which aliquots of the prepared solutions of the samples were titrated with indophenol solution to a light rose-pink color.

Antibiotic Properties.—The purpose of this study was to survey the plants under investigation for the presence of antibiotic substances which could form the basis of further research in the isolation and characterization of any active substances present. The plants were subjected to standard methods which have previously been used and found effective. No attempt was made to isolate or identify any antibiotic substances.

Since the plants used in this survey were brown algae, a method was chosen similar to that used on algae by Pratt, Daniels, and co-workers (8), who extracted chlorellin from a nutrient solution in which *Chlorella vulgaris* and *C. pyrenoidosa* had been grown.

In the following tests, the organism used was *B. subtilis* as a spore suspension in broth culture (9). The culture was made by seeding the broth with the bacteria and incubating seven to eight days with constant agitation. The resulting culture was pasteurized at 60° for thirty minutes resulting in a spore suspension that could be used to seed plates of nutrient agar. An approximately constant concentration of spores was maintained by using the same volume of culture to seed the agar plates. The cooled liquid nutrient agar was seeded with the broth culture, 25 cc. of broth to 200 cc. of agar, poured into Petri dishes, refrigerated one hour to set the plates, and then the various extracts from the plants were applied by soaking $^1/_2$-in. filter paper disks with the solution and applying three to each plate. The plates were incubated for twelve to fourteen hours at 37°, and then examined for appearance of zones of inhibition around the disks. The whole operation is essentially that of the filter paper disk penicillin assay method given by Foster and Woodruff (9). In each case suitable controls were set up using the extracting material. Tests were also made in the same manner with sea water obtained from the University of Washington Department of Oceanography and checks were run using penicillin with a strength of 100 units per cc.

The first test was run on the plants by taking the dried, ground plant and applying about 0.1 Gm. to the spore suspension agar plates. After incubation, the plates were examined for inhibition.

The next test was to treat 10 Gm. of the dried, ground plant with 100 cc. of water and 200 cc. of ammonium sulfate solution, heating the mixture to a temperature of 80° and filtering. After cooling, the *p*H was checked but not adjusted since in every case the solution was acid (*p*H about 6). The filter paper disks were then impregnated with the filtrate, placed on the seeded agar plates, incubated for twelve to fourteen hours, and examined for zones of inhibition.

The procedure employed by Berger (10) for purification of penicillin was used as the third method. An aliquot from 300 cc. of the filtrate, obtained as given above, was extracted with butyl alcohol, the butyl alcohol was then shaken out with petroleum ether, and then the petroleum ether was extracted with 2% sodium bicarbonate solution. Amounts were used in each step to give a ratio of 100 cc. of solvent to 100 cc. of extractive. The natural *p*H of the plants was so close to that recommended by Berger (*p*H 6.4) that no adjustment was made. The sodium bicarbonate solution was used to saturate the filter paper disks which were applied and read as before. The sodium bicarbonate solution was also concentrated to double strength by evaporation, *in vacuo*, at 60° and tested for its antibiotic properties.

The method of Pratt, Daniels, *et al.*, (8) was modified as follows: 100 cc. of ammonium sulfate solution was extracted with 150 cc. of chloroform in divided portions. The chloroform extracts were combined, evaporated *in vacuo*, the residue taken up in water and tested on the spore suspension agar plates. Chloroform was the only solvent used. The results of the various tests are shown in Table V.

In order to ascertain whether the inhibitory activity of *Cymathaere triplicata* was similar to that of known antibiotics and not due to some factor in sea water the following control tests were made: sea water with negative results and penicillin 100 units per cc. which produced 16- to 18-mm. zones of inhibition.

DISCUSSION

In the three kelps investigated, *Cymathaere triplicata*, *Hedophyllum sessile*, and *Egregia Menziesii*, algin, laminarin, and mannitol were found in amounts approximately equal to that of similar kelps. However, the quantities obtained can only represent minimum limits, since in any extraction procedures involving large amounts of crude material and reagents, under laboratory conditions, inevitable losses occur. Also, as already pointed out, a seasonal variation is to be expected in regard to the carbohydrate content. All of the plants were good sources of alginic acid, laminarin, and mannitol; although they are more difficult to gather than other species yielding comparable quantities of these constituents.

In investigating the vitamin content of these kelps, only the water-soluble vitamins were studied, since it was apparent from the absence of sufficient lipoids in these plants that useful concentrations of fat-soluble vitamins were not present. It is evident, however, that the plants are a rich source of vitamin B_1, niacin, and riboflavin. They also show a useful content of pantothenic acid, carotene, and vitamin C. The plants were collected in June and September and assayed in both the fresh and dried forms. No definite seasonal variation was indicated but it was shown that there was a high vitamin content at both seasons and that the vitamin content remained essentially high even after drying.

The results of the vitamin assays and ash determinations indicate the fact that these kelps would form an excellent adjunct to cattle food. Nor is it unreasonable to think that they might be valuable supplements in human diet if prepared in a palatable form. They might also be used as fertilizer though they are low in protein, but they would be valuable in rebuilding the mineral content of leached soils. These views are confirmed in similar studies by Lunde (11) on Norwegian marine brown algae. He found a lower content of thiamine in several plants studied and a higher content of ascorbic acid in fucus, than was found in the plants under consideration here.

Norris and associates (12) made a study of thiamine and ascorbic acid content of a large number of kelps collected from the same general area as those of this study. They found in general that these plants contained thiamine in amounts comparable to many fruits and vegetables. In their investigation of the vitamin C content of a number of kelps, *Egregia Menziesii* and *Hedophyllum sessile* were included. Their results are somewhat higher than those obtained in the present study. Their investigation did not include the determination of the thiamine content of these two plants.

In the antibiotic survey, only one of the plants, *Cymathaere triplicata*, gave positive results. Extractions of this plant consistently gave indications of antibiotic properties, which qualitatively resembled those given by penicillin G in a solution of 100 units per cc., when tested in the same manner as the plant. The fact that very little antibiotic potency is shown by the other plants is a point in favor of the conclusion that *Cymathaere triplicata* has definite antibiotic properties, since it tends to rule out the influence of extraneous factors which might be common to all such types of plants. In case of continued positive results with this plant, it would form the basis of a good chemical problem embodying the attempted isolation and characterization of the active substance or substances.

TABLE V.—RESULTS OF ANTIBIOTIC SURVEY OF THE KELPS

Kelp	Whole Plant	Ammonium Sulfate Extract	$NaHCO_3$ Extract, Unconcentrated	$NaHCO_3$ Concentrated, Double Strength	Method of Pratt, Daniels, *et al.*
Hedophyllum sessile	Slight inhibition	Negative	Negative	Negative	Negative
Egregia Menziesii	Negative	Negative	Negative	Negative	Negative
Cymathaere triplicata	Negative	Positive 18-mm. diameter zone	Slight inhibition	Positive 16-mm. diameter zone	Negative

SUMMARY

1. A partial proximate analysis and the mineral content of the plants have been made.

2. A determination was made of the three carbohydrates, alginic acid, laminarin, and mannitol.

3. The plant content of the water-soluble vitamins, carotene, ascorbic acid, thiamine, riboflavin, pantothenic acid, and niacin was determined.

4. In a survey of the plants, the presence of an antibiotic principle was indicated only in *Cymathaere triplicata*.

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Laboratory Assay of Clinically Effective Antiepileptic Drugs*,†

By EWART A. SWINYARD

Clinically useful antiepileptic drugs were assayed in rats for their ability to modify or prevent electrically or chemically induced seizures. The assays included modification of seizure pattern, ability to elevate normal and experimentally reduced electroshock threshold for minimal seizures, and antagonism to Metrazol. Toxic doses (TD_{50}) and protective doses (PD_{50}) were determined graphically and the protective indices calculated (TD_{50}/PD_{50}).

SINCE the introduction of diphenylhydantoin for the treatment of grand mal and psychomotor epilepsy, a large number of compounds have been tested for possible antiepileptic activity (1–5). Most of these drugs have been evaluated in laboratory animals either by their ability to raise the normal electroshock threshold or to prevent Metrazol and other chemically induced convulsions (1, 3, 5, 6). In many instances the experimental evaluation of antiepileptic activity has been made solely on the basis of a single criterion (3, 5, 6). It has become apparent that protective action against different epileptic manifestations (e.g., grand mal, petit mal) can be embodied in different drugs (7). Experimentally it has been shown that not all antiepileptic drugs have the ability to increase normal electroshock threshold, although anticonvulsant activity can be demonstrated by other electrical and chemical tests (8–12), and that not all antiepileptic drugs have protective value against Metrazol convulsions. It should be emphasized that up to the present time there is no single laboratory test available which in itself will definitively demonstrate the presence or absence of antiepileptic activity of a drug. For this reason it was thought advisable to integrate the most effective techniques known at present into a comprehensive routine assay procedure in such manner as to provide a means for rapidly screening and differentiating large series of new substances. Brief reports of the tests employed have been previously published (11–14).

* [illegible] n the College of Pharmac [illegible] City.
Pr [illegible] A. PH. A., San Francisco meeting, August, 1948.
† This [illegible] ted from the Division of Research [illegible] the National Institute of Health, U [illegible] e.

METHODS

From a considerable experience in screening large numbers of compounds for antiepileptic activity in various species, it has been found that the rat is the most convenient laboratory animal (11, 15). Sprague-Dawley male albino rats of uniform weight were used because they are easy to handle and respond uniformly. They were maintained on an adequate diet (Purina fox chow) and food was removed only during the actual assay.

For assays based on electrically induced convulsions, Spiegel (16) corneal electrodes were used and 60-cycle alternating current was delivered by means of an apparatus designed by Dr. L. A. Woodbury; the current was independent of the external resistance. Stimulus duration was 0.2 second. In all electroshock tests, the animal was restrained only by hand and released at the moment of stimulation in order to permit observation of the seizure throughout its entire course.

Whenever possible the drugs under investigation (sodium Mebaral, sodium phenobarbital, sodium diphenylhydantoin, sodium 5-thienyl-5-phenylhydantoin and trimethadione) were administered subcutaneously in aqueous solution. Compounds insoluble in water (Epidon, Phenurone) were suspended in 10% acacia solution and given orally. Drugs which were found to be poorly absorbed from the gastrointestinal tract (Mesantoin) or immiscible with water (Paradione) were dissolved in propylene glycol and given intraperitoneally. When propylene glycol was used, the concentration of drug was adjusted so that the maximum total dose of solvent administered did not exceed 1.5 cc. per kilogram All subcutaneous injections were made into a loose fold of skin in the midline of the back. Shoulder and pelvic girdle areas were avoided in order to eliminate sequelae of the injection which might erroneously be interpreted as neuromuscular deficit.

The various anticonvulsant tests were made at the previously determined time of peak drug effect, i.e., forty-five to sixty minutes for oxazolidine-2,4-diones and barbiturates, four to five hours for substituted 5-phenyl hydantoins, and three hours for Phenurone

General Procedure

The four standard tests used routinely are described below. Three of these tests are electrical and one is chemical. In addition, a toxicity determination is made. The tests employed are:

1. Maximal Electroshock Seizure (M.E.S.) Test.
2. Normal Electroshock Threshold (N.E.T.) Test.
3. Hydration Electroshock Threshold (H.E.T.) Test.
4. Metrazol Test.

The concentration of drug required to produce the desired effect in each test in 50% of experimental

animals (ED_{50}) is determined in two stages: (*a*) A series of five or six animals is given doses of the drugs to be tested in successive twofold steps until an effective dose range has been reached, i.e., the lowest dose which provides protection in each test and the highest dose which gives no protection. (*b*) Groups of 4 to 6 animals are then used for each of a series of dosages within the indicated effective range, and the percentage of animals protected in each group determined. The percentages are converted to probit *values and plotted against log concentration. The* effective dose (ED_{50}) and its standard error are determined graphically by the method of Miller and Tainter (17). Finally, the dose which is toxic to 50% of animals (TD_{50}) is divided in turn by each of the ED_{50} values for the four anticonvulsant tests, to give four protective indices.

To calculate the standard error of each protective index, the two determined standard errors (of the toxic and effective doses) were converted to fractional parts of their respective values, and the root mean square of these fractional values was then multiplied by the protective index. The standard errors so obtained are first approximations and should not be used for critical comparisons of the significance of the results.

Standard Tests

In each of the following tests, care is exercised to exclude animals which have not fully recovered from the effects of previously administered drugs. The tests may be combined in various ways in order to conserve animals; for example, toxicity data may be obtained from the same animals used in the M.E.S., N.E.T., or Metrazol test.

(1) **Maximal Electroshock Seizure (M.E.S.) Test.**—Animals are given a single supramaximal shock with a current intensity of 150 mA., which is *approximately 5 times threshold. All control ani*mals should exhibit a definite extension of the hind legs during the tonic phase of the seizure, and aberrant animals not manifesting tonic extension are not employed for this test. Abolition of this extensor component after drug treatment is taken as the end point of this test (11–15, 18).

(2) **Normal Electroshock Threshold (N.E.T.) Test.**—For initial determination of control threshold for minimal electroshock seizures, shocks of 0.2 second duration are given at intervals of six to eight hours, with small (3%) increments or decrements in current intensity until a "minimal" seizure occurs. In rats, minimal seizures are defined as those which consist of ten or more seconds of facial clonus without loss of righting reflexes. With this criterion the normal threshold for minimal electroshock seizures (159 rats) varies between 20 and 36 mA. (average 28 ± 0.29 mA.), and a change of 5% in threshold can be detected without difficulty. For any one animal, the threshold is quite constant. However, it does vary from rat to rat with age, nutritional state, environmental and body temperature, etc. (19, 20). For determination of drug action, a single shock having a current intensity 20% above control threshold is used. Absence of even a minimal seizure is taken as the end point of this test.

(3) **Hydration Electroshock Threshold (H.E.T.) Test.**—For control determinations, the minimal electroshock seizure threshold is determined as before. Four hours before the anticipated time of peak drug effect, each animal is injected intraperitoneally with 10 cc. of freshly prepared isomolar (5.5%) glucose solution per 100 Gm. body weight. It has previously been shown (9) for a large series of animals that four hours after injecting the glucose solution, the electroshock threshold is reduced to 44 ± 1.26% of its initial value. To test drug effectiveness in preventing this degree of reduction in threshold, a single test shock is given having a current intensity 66% of the normal electroshock seizure threshold for each animal. This current intensity represents a *50% increase above the experimentally lowered* hydration threshold. Absence of even a minimal seizure is taken as the end point of this test.

(4) **Metrazol Test.**—It has previously been determined that a subcutaneous dose of 70 mg./Kg. of Metrazol (5% aqueous solution) will cause seizures in more than 97% of rats (CD_{97+}) (13). This amount is approximately twice the threshold dose for 50% of animals. Because of the high percentage of deaths with this dosage, control determinations are usually omitted. The test is carried out by making the Metrazol injection approximately ten minutes before anticipated time of peak anticonvulsant drug action and observing the animals during the following sixty minutes for the occurrence of seizures. A threshold convulsion is defined as one episode of clonic spasms which persists for at least a five-second period. Transient intermittent jerks or tremulousness are not considered as constituting a convulsion. Absence of even a threshold convulsion during an hour of observation is taken as the end point of this test.

Toxicity Determination.—The following 3 criteria were found most useful for disclosing minimal neurological deficit at the time of peak drug action.

Walking and Postural Test.—Neurological deficit is indicated by a circular or zig-zag gait, ataxia, *abnormal spread of the feet, absence of postural re*flexes, hyperactivity, stupor, catalepsy, etc.

Vertical Screen Test.—The normal rat will climb on a vertical screen ($^1/_4$-in. mesh) and only occasionally lose its footing. Neurological deficit is indicated by a tendency to slip or inability to maintain its position on the screen.

Positional Sense Test.—If the hind leg of a normal rat is gently lowered over the edge of a table, it will be quickly lifted back to a normal position. Neurological deficit is indicated by inability rapidly to correct such an abnormal position.

Deficit disclosed by any of these three examinations for evidence of drug toxicity was taken as the end point. However, if other side effects consistently appeared at lower doses (hematuria, hyperexcitability, hyperpnea, etc.), these were taken as the end point.

RESULTS

In order to establish the validity of laboratory tests for predicting clinical specificity and potency of new anticonvulsant drugs, the clinically useful antiepileptics were assayed by the procedures outlined. The results are shown in Table 1 and Fig. 1.

Substituted 5-phenyl hydantoins (diphenylhydantoin, Mesantoin, 5-phenyl-5-thienylhydantoin) useful in grand mal are characterized in the laboratory by a high protective index by the maximal electroshock seizure test. A high protective index by the normal electroshock threshold or Metrazol test does

TABLE I.—PROTECTIVE INDICES OF CLINICALLY USEFUL ANTIEPILEPTIC DRUGS BY FOUR DIFFERENT ASSAY METHODS IN RATS

Drug	TD_{50}, Mg./Kg.	Protective Indices[a]: Maximal Electroshock Seizure Pattern	Normal Electroshock Threshold	Hydration Electroshock Threshold	Metrazol Seizure Protection	Major Usefulness in Epilepsy
Mesantoin	50 ± 3.1	11.1 ± 0.71	0.69 ± 0.07	1.42 ± 0.25	0.91 ± 0.08	GM PsM
5-Thienyl, 5-phenyl hydantoin	75 ± 6.5	4.47 ± 0.42	0	0.53 ± 0.05	0	GM
Diphenyl-hydantoin	104 ± 8.0	2.36 ± 0.24	0	1.1 ± 0.09	0	GM PsM
Phenobarbital	22 ± 1.1	3.58 ± 0.26	1 75 ± 0.19	1.18 ± 0.12	0 85 ± 0.08	GM
Mebaral	38 ± 1.1	2.46 ± 0.08	1.28 ± 0.20	2.04 ± 0.16	2.51 ± 0.18	GM
Trimethadione	445 ± 27.7	1.23 ± 0.11	1.51 ± 0.17	1 17 ± 0.13	1.48 ± 0.13	PM
Paradione[b]	167 ± 11.5	0.7 ± 0.08	1.11 ± 0.10	1.05 ± 0.11	1 22 ± 0.14	PM
Epidon[c]	1200 ± 54.2	1.78 ± 0.25	1.00	1.00	0	GM
Phenurone[d]	730 ± 99.2	17.7 ± 2.60	18.7 ± 3.70	3.24 ± 0 72	3 65 ± 1 1	GM PsM PM

[a] For details of assay procedures and end points employed, see text. GM, grand mal; PsM, psychomotor; PM, petit mal. ±S.E.
[b] 3,5-Dimethyl-5-ethyl oxazolidine-2,4-dione.
[c] 5,5-Diphenyl-oxazolidine-2,4-dione.
[d] Phenacetylurea.
Phenurone, Trimethadione, and Paradione were supplied by Abbott Laboratories; Mebaral by Sterling-Winthrop; Mesantoin by Sandoz Chemical Works; and 5-thienyl, 5-phenyl hydantoin by Eli Lilly & Co

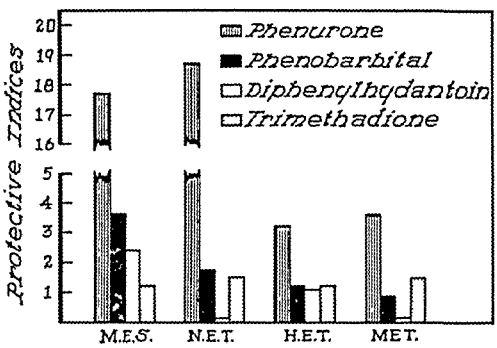

Fig. 1.—Profiles of Action of Anticonvulsant Drugs in Rats. "M.E.S.," maximal electroshock seizure test. "N.E.T.," normal electroshock threshold test. "H.E.T.," hydration electroshock threshold test. "MET.," Metrazol seizure test. Note the break in the ordinate scale of protective indices.

not appear to be an obligatory feature of this chemical nucleus. It is noteworthy that the substituted 5-phenyl hydantoins elevate the experimentally lowered threshold even though they fail to elevate significantly the normal electroshock threshold.

Barbiturates (phenobarbital, Mebaral) effective in grand mal show activity by all four assay procedures. They are particularly characterized by their ability to modify maximal seizure pattern and contrast sharply with the substituted 5-phenylhydantoins in their ability to elevate normal electroshock and Metrazol thresholds.

Oxazolidine 2,4-diones (trimethadione, Paradione), useful in petit mal, rank between the barbiturates and substituted 5-phenyl hydantoins by the normal and hydration electroshock threshold tests, and are a poor fourth by the maximal electroshock test. Metrazol antagonism is the most significant feature of these compounds and in this respect contrasts sharply with Epidon which is ineffective against Metrazol. It is of interest to note that Epidon, also an oxazolidine 2,4-dione, resembles the substituted 5-phenyl hydantoins when assayed by these tests. Likewise, it is clinically more useful in grand mal than in petit mal.

Phenacetylurea (Phenurone) shows marked activity by all four tests. Indeed, Phenurone has the highest index by all four assay procedures of any compound yet tested. In this regard it is interesting to note that preliminary clinical trial indicates it is also effective against all three major seizure types (21).

DISCUSSION

Figure 1 shows the profile of action of four representative drugs, Phenurone, phenobarbital, diphenylhydantoin, and trimethadione, when assayed by the procedure discussed above. It is obvious that each test gives a different evaluation of anticonvulsant drug action, and that simple elevation of threshold is not alone adequate as a criterion of antiepileptic activity. For example, diphenylhydantoin neither raises threshold for minimal electroshock seizures nor prevents Metrazol convulsions. A potent and valuable antiepileptic like diphenylhydantoin could thus be missed if evaluated only by these tests. On the other hand, diphenylhydantoin elevates the experimentally lowered threshold for minimal seizures and markedly alters the pattern of maximal electroshock convulsions. The sharpest difference lies between the results of the maximal electroshock seizure and Metrazol tests. For this reason, and because they are rapid and easy to perform, these two tests are used for the preliminary screening of new drugs. However, no one laboratory test is sufficient to measure the potential anticonvulsant potencies of a new drug. Therefore, more promising compounds should be subjected to the remaining tests in order more definitely to demonstrate their anticonvulsant activity.

SUMMARY

Clinically useful antiepileptic drugs are assayed in rats for their ability to modify or prevent elec-

trically or chemically induced seizures. The assay methods include modification of seizure pattern, ability to elevate normal and experimentally reduced electroshock threshold for minimal seizures, and antagonism to Metrazol. Toxic doses (TD_{50}) and effective doses (ED_{50}) are then determined graphically and the protective indices calculated (TD_{50}/ED_{50}). The details of a routine assay procedure are described.

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Preliminary Report on the Volatile Oil of *Pycnanthemum Pilosum* Nutt.*,†

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A report on the physical and chemical constants of the oil is presented

INTRODUCTION

THE plant material for this study was obtained from the vicinity of Atlantic, Iowa, through the courtesy of Mr. Frank Pellett, who is interested in the cultivation of the plant for bee pasture. The 1945 crop was represented by 154 pounds of material harvested from an area of 432 square feet representing a yield of 15,500 pounds per acre. Distillation of the fresh plant material yielded 629 grams of deep yellow-colored oil which separated from the aqueous distillate, and cohobation yielded an additional 43 grams. The total yield of oil corresponded to 0.976 per cent of the fresh plant material.

The crops for 1946 and 1947 averaged 17,500 pounds per acre, with an oil yield of 0.95 per cent.

EXPERIMENTAL

Physical Properties.—All of the oil samples were yellow in color, but there was some variation in intensity, with the 1945 oil being the darkest. The odor was sweet and strong, suggesting the presence of pulegone. The physical constants, determined at 25°, are recorded in Table I.

Attempts to cause the oil to congeal or to yield a crystalline component were unsuccessful, even at a temperature of −60°.

Saponification Value.—The saponification values and an interpretation of them for the various oil samples are given in Table II.

No explanation is offered for the variations in the values obtained. The 1945 oil showed an unusually high solubility value, and originally it was felt that the cohobation process might have been conducted in a manner such that the recovery of water-soluble acids was high; however, the acid values of all of the oil samples were negligible.

To interpret the saponification value in terms of the alcohol in the original oil (Table II) it was assumed that the alcohol present is one of the isomeric forms of $C_{10}H_{19}OH$ and, to estimate the percentage of acetate ester, it was assumed that this alcohol occurs as the acetic acid ester (1).

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† Abstracted from theses submitted by Victoria Chilquist and Robert Gilmour for the degree of Bachelor of Science.

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TABLE I.—PHYSICAL CONSTANTS

	1945 Oil		1946 Oil	1947 Oil	
	Original	Cohobate		Original	Cohobate
Specific Gravity	0.9225	0.9483	0.9378	0.9233	0.9328
n_D^{25}	1.4690	1.4750	1.4795	1.4787	1.4708
$[\alpha]_D^{25}$	11.9	16.8	14.5	12.7	18.2
Solubility Value	560	658	...	...	...

TABLE II.—SAPONIFICATION VALUES

Sample	Sapon. Value	Per Cent Alcohol in Original Oil	Per Cent Acetate Ester
1945 original oil	38.0	10.90	13.44
1945 cohobated oil	82.4	24.34	28.99
1946 original oil	30.4	8.55	10.61
1947 original oil	15.5	4.37	5.48
1947 cohobated oil	12.8	3.60	4.52

Total Alcohol and Free Alcohol.—Duplicate samples of the original oil (1945) were acetylated in the usual manner, and the average saponification value of the acetylated oil was found to be 72.40. This value corresponds to 21.35% of total alcohol ($C_{10}H_{19}OH$) in the oil. Therefore, the calculated amount of free alcohol is 10.45%.

In like manner, the saponification value of the acetylated cohobated oil (1945) was found to be 98.00. This value corresponds to 27.3% of total alcohol ($C_{10}H_{19}OH$). Therefore, the calculated amount of free alcohol in this instance is 4.90%.

Acetyl Value.—Based on the saponification values of the acetylated samples, the acetyl value of the original oil (1945) was calculated to be 1.28, while that of the cohobated oil (1945) was 1.75.

Phenol Determination.—Carvacrol has been reported to occur in the volatile oil of *Pycnanthemum lanceolatum*, ranging from 7 to 58% (2). The phenol content of *P. pilosum* oil was determined, using 10-cc. samples, and found to be as follows:

1946 oil.....7.5%
1947 oil.....3.1%
1947 oil (cohobated).....4.6%

The free phenols were isolated from the alkaline aqueous solutions by acidification with hydrochloric acid and extraction with ether. After the ethereal solutions had been dried over anhydrous sodium sulfate and the solvent had been removed, the slight, brown, oily residues remaining represented the phenol fraction of the oils. All of the residues so obtained yielded a positive Flueckiger reaction (tan coating on NaOH pellet) and a brown color with ferric chloride solution, following the application of heat.

An attempt was made to prepare the phenyl urethane of the phenol, but without success. Even though the hydrocarbon solvent was dried over sodium, sufficient moisture was present to form the di-substituted urea which melted at 233°. Carvacrol phenyl urethane melts at 137°.

Ketone Determination.—The presence of pulegone was suggested by the odor of the various oils. Several methods were applied for the determination of the aldehyde and ketone content, but the results were not comparable, due to the pulegone being present. For example, the hydroxylamine methods (3) which were very slow, yielded high results because pulegone is capable of reacting with two moles of the reagent. Apparently, however, the reaction involving the addition of hydroxylamine at the point of unsaturation is not quantitative. The most consistent results were obtained by the sodium sulfite method. They are as follows:

1945 oil.....51%
1946 oil.....37%
1947 oil.....33%
1947 oil (cohobated).....21%

The ketone fraction was isolated from the assay solutions after the unreacted oil had been separated. These clarified aqueous solutions were made strongly alkaline with sodium hydroxide, and then steam distilled. The small quantities of oily ketone which separated were isolated and dried over anhydrous sodium sulfate. The physical constants for the ketone fractions are listed in Table III.

TABLE III.—PHYSICAL CONSTANTS FOR KETONE FRACTIONS

Sample	Specific Gravity	Refractive Index
1946 oil	0.9334	1.486
1947 oil	0.9319	1.488
1947 oil (cohobated)	0.9309	1.488

The similarity of these constants suggested the common identity of the water-insoluble ketone fractions, therefore they were combined and the optical rotation was measured. $[\alpha]_D^{25} = 22.25°$. The normal oxime melted at 124°. The physical constants for the ketone and its oxime are in agreement with those for pulegone.

Aqueous Cohobate.—In the initial distillation of the oil (1945), approximately 3 gallons of distillate were collected. Following the separation of the oil, the aqueous portion was cohobated. When the original volume had been concentrated by this process to 500 cc., the oily layer was removed, and the aqueous portion was further concentrated, but no more oil separated. The concentrate possessed a strong, honey-like odor, and this together with the high solubility value reported earlier indicated that there were present in the oil appreciable quantities of water-soluble components. The concentrate was examined for the presence of alcohols, aldehydes or ketones and acids.

A sample reacted positively with Fehling's and Tollen's reagents and with fuchsin-sulfite solution, indicating the presence of reducing substances like aldehydes and ketones. A 10-cc. portion of the

solution was neutralized by 0.437 cc. of 0.1 *N* sodium hydroxide solution. Calculated as acetic acid, this corresponds to 0.026%, indicating a negligible acid content. The Schotten-Baumann reaction, when applied to the sample, yielded a few drops of oily benzoyl ester, b. p. 135°, $n_D^{20} = 1.4670$. The yield was too small for additional data.

SUMMARY

In a preliminary report, the physical and chemical constants for the volatile oil of *Pycnanthemum pilosum* are given for samples obtained during three successive years. The presence of phenols is indicated, and pulegone was isolated and identified. The aqueous cohobate was shown to contain an alcohol and an aldehyde or ketone.

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A Comparative Study of the Effect of Various Solutions on the Phosphorus Depletion of the Rat Tooth Using Radioactive Phosphorus*,†

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Rat incisors become radioactive when labeled phosphorus is injected intraperitoneally. A new technique using radioactive isotopes as tracer elements in the study of the effect of various solutions on the teeth is described. Applications of the method are stressed.

ONE OF OUR greatest problems of the present day is the care and protection of teeth. Poor teeth represent the most common disorder of civilized man; more than 90 per cent of all persons have tooth decay or other diseases of the mouth at some time or another during their lives (1). At a time when medical science has triumphed over many infectious diseases, tooth decay presents problems which have baffled the dental and medical professions. One of the main problems of commercial concerns is the difficulty which they encounter in testing the action and effect of their products on the teeth. It is extremely difficult to test these preparations using the techniques described in the literature.

Perhaps the most important observation was made by Cox (2) in a review article in which he stated, "investigators, working with teeth, have no satisfactory method for reporting data." Some methods reported in the literature for determining the action of solutions on teeth consist of data based on the loss of weight of a tooth after immersion in solutions (3), examination of teeth with a reflecting microscope (4, 5), examination of photomicrographs of ground longitudinal sections (6), and by scratching the tooth with a sharp-pointed instrument by which teeth showing softening were classified as decalcified (7). These methods are reported to be somewhat unsatisfactory.

In recent years the use of radioactive tracers in animal and human metabolism has come into prominence. Greenburg (8) reported that Chievitz and Hevesy in some of their first experiments found radioactive phosphorus in the teeth of animals after administration. This suggests the possibility of the use of radioactive isotopes in the study of teeth.

Following the early work of Hevesy in which he showed that radioactive phosphorus was deposited in teeth, various workers have used this tracer element in studying the metabolism of laboratory animals. Manly and Bale (9) using P^{32} found rats' incisors showed a 2.0–3.5 per cent uptake of P^{32} in four hours and 8 per cent uptake

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in twenty days. Hevesy and Armstrong (10) reported that *in vitro* experiments showed the P^{32} acquired by enamel *in vivo* is apparently not delivered from saliva.

Hurley (11) showed that the most economical and efficient method for causing rat teeth of a heterogeneous group of rats to become radioactive with radioactive phosphorus to such an extent that the teeth contain at least 35 counts per minute per mg. (as measured 1.3 cm. from an Eck and Krebs counter tube) is to inject the rats intraperitoneally with a radioactive potassium phosphate solution containing between five and seven million counts per minute per cc. and remove the teeth twenty hours after injection. There are various other workers who have shown the uptake of labeled phosphorus in the teeth of various animals (12–21).

The literature offers no effective quantitative method for the demonstration of the effects of various solutions on the mineral depletion of teeth. One of the purposes of this work was to develop a method using a radioactive isotope as a tracer element by which the mineral depletion of teeth could be determined. Such a technique is described in the following experimental section and should prove to be of considerable value in the study of normal and abnormal metabolism of teeth where a precise method capable of detecting small differences in mineral content of the teeth is desirable.

EXPERIMENTAL

Before carrying out experiments involving the up take and depletion of phosphorus in rats' teeth using radioactive phosphorus as a tracer element, it is desirable to know the mode of entry of phosphorus into the tooth. If considerable amounts of labeled phosphorus are adsorbed on the tooth from the saliva the interpretation of results would be quite different than if labeled phosphorus enters the tooth through the blood stream.

An experiment was carried out to determine if phosphorus entered the rat tooth through the saliva. The upper teeth were covered with a metal cap and the lower teeth were left exposed to the saliva. Since it was necessary to have the teeth covered for a period of twenty hours, a "muzzle" was designed from discarded X-ray film to prevent the rat from removing the metal cap. Two holes were made in the film for the rat's front legs, and the rat was placed in the muzzle. The film was brought up over the rat's head and stapled close to the body. This is a convenient, rapid method for restricting the animal movements as desired, and may be used for other experiments where restriction of the animal is necessary.

The labeled phosphorus uptake was the same in the capped and uncapped teeth indicating that no phosphorus was taken up from the saliva.

The effect of various solutions on the labeled phosphorus content of rats' incisors was carried out on a qualitative basis as a preliminary to quantitative determinations. Radioactive teeth extracted from albino rats were subjected to various solutions and the activity of the solutions determined, indicating the extent of phosphorus depletion. The results obtained are summarized in Table I.

The procedure for the quantitative determinations of the labeled phosphorus depletion of rats' teeth when exposed to various solutions is described in detail as follows:

A known quantity of radioactive potassium biphosphate (KH_2PO_4), weighed accurately, was dissolved in 100 cc. of distilled water. This solution was standardized and the time and date of standardization noted.

Albino rats of medium size were injected intraperitoneally with that amount of KH_2PO_4 which had an activity of approximately 5,000,000 counts/min./cc. The rats were sacrificed after twenty hours by the use of chloroform. They were decapitated with a pair of scissors and the heads boiled in tap water for ten to fifteen minutes. The heads were removed from the boiling water and the incisors extracted. The teeth from each rat were placed in a separate beaker and 10 ml. of 0.05% KOH was added and the solution brought to a boil to clean and remove all extraneous material. The teeth were removed immediately, washed with tap water until free of KOH (about 5 washings), washed once with distilled water, and placed on a filter paper to air dry.

One of the four incisors from each rat was used as a standard. This tooth was ground to a fine powder in a Wedgwood mortar and 5.0 mg. of the powdered tooth was placed in a Kimbleware cup. One drop of 20% sulfuric acid was added and the contents of the cup charred in a hot-air oven (145°) after which the contents of the kimbleware cup were ashed in a muffle furnace at 500–550° and checked for radioactivity by the Geiger-Müller Counter.

The other three incisors were treated in the following manner before being placed in the test solution. Between one-eighth and one-fourth inch, depending on the size of the tooth, was removed from the root end of each tooth by using an electric dental saw. The root end of the tooth was then buffed until the teeth were all the same weight when checked on a Torsion balance. The ends of the teeth were covered with shellac; care was taken that no shellac was placed on the body of the tooth. After the shellac dried, each tooth was placed in the test solution and left for seventeen hours. Six-inch Pyrex test tubes were used to hold the solution which was held in a constant temperature bath (37.5°). The test tubes were stoppered to prevent evaporation. After seventeen hours the teeth were removed from the test solution. The test solution was evaporated in kimbleware cups to dryness by placing the cups in a hot-air oven. One drop of 20% sulfuric acid was added and the contents of the cups ashed in a muffle furnace at 500–550°. The ash was then checked for radioactivity by the Geiger-Müller Counter.

In a recent article Wernimont (22) reviewed the use of control charts. A control chart may be described as a graphic presentation of test data in such a manner that the variability of all the results is compared with the average variability within (arbitrary) small groups of test results. The "control"

TABLE I.—SUMMARY OF DATA OF QUALITATIVE DETERMINATIONS OF THE PHOSPHORUS DEPLETION OF RATS' TEETH WHEN EXPOSED TO VARIOUS SOLUTIONS

Solution Tested	*p*H	Number of Teeth Used[a]	Average Activity per 5-Cc. Sample in Counts/Min.
Product A[b]	2.95	60	18.7
Product A	2.95	11	12.2
(Teeth previously exposed to Product A for ten hours)			
Product A (colorless)	2.95	16	41.8
Tap water	6.60	14	35.3
Normal saline solution (0.9% NaCl)	6.90	23	60.1
Saccharin..... 0.036 Triple-distilled water, *q. s.*.....100 cc.	2.75	23	254.8
Saccharin..... 0.036 Zinc chloride..... 0.219 Triple-distilled water, *q. s.*.....100 cc.	2.95	23	203.9
Saccharin..... 0.036 Zinc chloride..... 0.219 Absolute alcohol..... 5.0 Triple-distilled water, *q. s.*.....100 cc.	2.65	23	53.2
Buffer Solution U. S. P.	1.15	4	3292.5
Buffer Solution U. S. P.	1.95	5	961.5
Buffer Solution U. S. P.	3.00	4	840.2
Buffer Solution U. S. P.	4.05	5	617.0
Buffer Solution U. S. P.	4.85	5	465.0
Buffer Solution U. S. P.	5.95	5	173.0
Buffer Solution U. S. P.	7.00	6	68.8
Buffer Solution U. S. P.	7.80	6	53.9
Buffer Solution U. S. P.	8.75	1	35.5
Buffer Solution U. S. P.	9.60	1	30.5
Zinc chloride..... 0.219 U. S. P. Buffer (*p*H 3.00), *q. s.*...100 cc.	2.90	5	442.6
Zinc chloride..... 0.219 U. S. P. Buffer (*p*H 3.00), *q. s.*...100 cc.	2.90	5	521.6
(Teeth previously exposed to solution for ten hours)			
Saccharin..... 0.036 U. S. P. Buffer (*p*H 3.00), *q. s.*...100 cc.	2.90	5	300.6
Saccharin..... 0.036 U. S. P. Buffer (*p*H 3.00), *q. s.*...100 cc.	2.90	5	291.0
(Teeth previously exposed to solution for ten hours)			
Saccharin..... 0.036 Zinc chloride..... 0.219 U. S. P. Buffer (*p*H 3.00), *q. s.*...100 cc.	2.95	5	488.8
Saccharin..... 0.036 Zinc chloride..... 0.219 U. S. P. Buffer (*p*H 3.00), *q. s.*...100 cc.	2.95	5	561.0
(Teeth previously exposed to solution for ten hours)			
Absolute alcohol..... 5.0 cc. Triple-distilled water, *q. s.*.....100 cc.	8.00	16	556.5
Absolute alcohol..... 5.0 U. S. P. Buffer (*p*H 1.95), *q. s.*...100 cc.	2.20	7	441.3
Triple-distilled water	6.20	47	76.0
Triple-distilled water	6.20	7	45.0
(Teeth previously exposed to solution for ten hours)			

[a] The teeth were placed in each solution for a period of ten hours.
[b] Commercial mouthwash.

shown by the control charts formulated from the results obtained by this technique was excellent taking into consideration the variability of a biological animal such as the rat.

The qualitative results indicate that the *p*H of a solution may have considerable effect upon the phosphorus depletion of the teeth; however, the quantitative results indicate that the *p*H is not the only factor that effects the removal of phosphorus. Triple-distilled water, having a *p*H of 6.15, removed an average of 3.16%, whereas tap water with a *p*H of 7.05 removed 0.98%, and a commercial mouthwash with a *p*H of 2.95 removed only 0.92%. Therefore, factors other than *p*H have an effect upon phosphorus depletion. It was found in these experiments that the combination of absolute alcohol along with deionized water had an effect in preventing phosphorus depletion in rats' teeth *in vitro*.

The commercial mouthwashes tested removed in each case somewhat less phosphorus than does triple-distilled water and in most cases slightly more than tap water.

It was of considerable interest to test a cola beverage for phosphorus depletion by this technique since a large amount of soft drinks are consumed each year. The particular product tested removed approximately twice as much phosphorus as triple-distilled water and approximately five to seven times as much as the commercial mouthwashes tested.

Since candies have been reported to be detrimental to the teeth and since most candies contain glucose, it was of interest to determine the phosphorus deple-

TABLE II.—SUMMARY OF DATA OF QUANTITATIVE DETERMINATION ON THE PHOSPHORUS DEPLETION OF RATS' TEETH WHEN EXPOSED TO VARIOUS SOLUTIONS

Solution Tested	pH	Number Teeth Used	Per Cent Labeled P Removed	Standard Error
Tap water	7.05	54	0.98	0.033
Triple-distilled water	6.15	72	3.16	0.135
Zinc chloride..... 0.219				
Saccharin..... 0.036				
Triple distilled water, *q. s.*.....100 cc.	3.05	66	3.62	0.145
Zinc chloride..... 0.219				
Saccharin..... 0.036				
Absolute alcohol..... 5.0				
Triple-distilled water, *q. s.*.....100 cc.	2.95	72	2.16	0.071
Zinc chloride..... 0.219				
Saccharin..... 0.036				
Absolute alcohol..... 5.0				
Deionized water, *q. s.*.....100 cc. (as used in preparation of Product A)	2.91	72	0.83	0.039
Product A[a]	2.95	72	0.92	0.052
Product B[a]	3.55	72	1.51	0.079
Product C[a]	4.65	72	1.14	0.086
Product D[a]	3.25	72	2.69	0.091
Product E[a]	7.25	69	2.96	0.271
Antiseptic Solution N. F.	5.35	66	0.97	0.085
Cola Beverage	2.50	63	6.28	0.147
Glucose 5% (distilled water solution)	4.90	63	3.88	0.085
Lemon juice	2.29	57	71.74	1.880
Grapefruit juice	3.25	60	38.97	0.967

[a] Commercial mouth washes.

tion of teeth in a 5% glucose solution in distilled water. The result compared closely to that obtained with triple-distilled water alone.

Approximately 70% of the labeled phosphorus in rats' incisors was removed by the action of lemon juice. This compares with the clinical findings of reports on the demineralization effects of lemon juice on human teeth (23). Grapefruit juice was found to remove approximately 40% of the labeled phosphorus from rats' incisors.

It must be emphasized that the results obtained in this work do not indicate the amount of phosphorus removed under normal conditions of usage of these substances *in vivo*, but are relative values based on *in vitro* tests which indicate that certain substances have greater ability to cause phosphorus depletion as compared to other substances under the conditions of the experiment.

In order to obtain significant results in these experiments, a total of approximately 2500 rat teeth representing approximately 1000 rats were used. Approximately four hundred and twenty hours were required to determine the radioactivity of various samples using the Geiger-Müller Counter.

SUMMARY

When labeled phosphorus is injected intraperitoneally into rats the rat incisor becomes radioactive. It has been shown that none of the phosphorus is adsorbed on or absorbed through the normal tooth enamel from the saliva. The labeled uptake of phosphorus was the same in capped and uncapped teeth.

A new convenient rapid technique for restricting the animal movements, using discarded X-ray film, has been described.

A new technique using radioactive isotopes as tracer elements in the study of the effect of various solutions on the teeth has been described. This technique has wide applications and has been used in this work to study the effect of certain mouthwashes, beverages, fruit juices, triple-distilled water, and tap water on the phosphorus depletion of rat teeth. Although the phosphorus depletion of teeth is not as important as the calcium depletion, the results obtained with phosphorus are perhaps indicative of calcium depletion since, although not entirely proved, most investigators feel that the calcium and phosphorus are in chemical combination in the tooth. The technique, however, may be used equally as well with the study of calcium or strontium depletion of the teeth since radioactive strontium and calcium are now available.

Experimentation is being continued on the problems involved with the effect of various solutions on mineral depletion of teeth with a view to improve the techniques involved and to obtain further information concerning the mechanism of depletion.

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Studies on Pharmaceutical Powders and the State of Subdivision. I. The Application of Low-Temperature Nitrogen Adsorption Isotherms to the Determination of Surface Areas*,†

By J. V. SWINTOSKY,‡ S. RIEGELMAN,§ T. HIGUCHI and L. W. BUSSE

Although it has been shown qualitatively that the pharmacological effects of sparingly soluble drugs are strongly influenced by the fineness of these pharmaceuticals, very little in the nature of quantitative study of this phenomenon has been attempted. A project has been initiated to correlate pharmacologic effects of slightly soluble pharmaceutical powders with their specific surface areas. In the present paper, which is the first of a series proposed in this program, the practicability of the nitrogen adsorption method for the determination of specific surface areas of pharmaceutical powders is shown. The significance of particle dimensions as calculated by these results is also discussed. Data are presented illustrating the application of the method to representative samples of titanium dioxide, zinc oxide, and barium sulfate.

THE PRESENT INQUIRY into the state of subdivision of pharmaceutical powders was initiated for a number of reasons. Precise standards have not been established for particle size and surface areas of pharmaceutical powders and as a consequence the size and surface relationships of presently available powders are not well known. There appear to be significant relationships between surface characteristics of powders and their physiological activities, yet only few quantitative correlations of these properties have been made. Also in the technology of pharmaceuticals surface and size characteristics obviously play an important role; however, little emphasis has ever been placed on uniformity and precise particle size values for this purpose.

Surface properties, especially specific surface areas,[1] are particularly important in slightly soluble substances since they are factors in solution rate, solubility, and absorption. It is then reasonable to assume that therapeutic response and antiseptic activity are closely related to the surface characteristic of those drugs which fall into the category of being slightly soluble. Some evidence to this effect has already been presented by a number of workers during the course of investigations on such substances as sulfadiazine (1), sulfathiazole (2–4), sulfur (5–8), phenolphthalein (9), mercury compounds (10, 11), and certain insecticides (12–18). From the standpoint of pharmaceutical technology, the role of particle dimensions in suspensions, emulsions, lotions, magmas, ointments, and similar preparations is important in achieving stability and elegance preparations of these types.

Comprehensive studies on the physical characteristics of pharmaceutical powders would then appear to serve a useful purpose. These studies should employ sufficiently precise analytical methods so that the data obtained could be applied to therapeutic and pharmaceutic investigations where the results could be interpreted quan-

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† This paper is based in part on a thesis submitted by Joseph V. Swintosky to the Graduate School of the University of Wisconsin in partial fulfullment of the requirements for the degree of Doctor of Philosophy.
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[1] The specific surface area of a powder is the sum of the surface areas of all the particles in a gram of the powder. It is an intensive property of the powder similar to density, specific heat capacity, etc.

titatively according to well-known physical and chemical laws.

A number of methods for the determination of size and surface characteristics of powders are available. The purpose to which such data are to apply would govern to some extent the choice of analytical procedure to be followed. Some of the more important methods of powder analysis are listed below (21).

1. Sieve Analysis
2. Microscopic Analysis
   A. Simple Microscopic Methods
   B. Ultramicroscopic Methods
   C. Electron Microscopic Methods
3. Sedimentation Analysis
   A. Increment Methods
      (a) Pipette Method
      (b) Photographic Method
      (c) Pressure Method
      (d) Hydrometer Method
   B. Cumulative Methods
      (a) Balance Methods
      (b) Pressure Methods
4. Centrifugal Analysis
   A. Ordinary Centrifugal Methods
   B. Supercentrifugal Methods
   C. Ultracentrifugal Methods
5. Elutriation Analysis
   A. Air Elutriator Methods
   B. Liquid Elutriator Methods
6. Turbidimetric Analysis
   A. Gross Methods
   B. Size Distribution Methods
7. Miscellaneous Methods of Analysis
   A. Permeability Methods
   B. Energy of Emersion Methods
   C. Adsorption Methods

Criteria for selection of an analytical method and descriptions of such methods are available in standard references on this subject.

In the classification given above, permeability methods, energy of emersion methods, and adsorption methods constitute procedures for the absolute measurement of specific surface areas. An adaptation of the permeability method has seen application for this purpose (23) in the study of some pharmaceutical powders. The energy of emersion method requires very elaborate equipment and refined techniques not suitable for routine analysis of powders. The adsorption method was chosen for the present study because of its relative simplicity and proved utility.

This paper will be confined to an analysis of several pharmaceutical powders by gas adsorption. With this method it is possible to determine the type of van der Waal adsorption exhibited by each particular substance, the absolute surface areas may be ascertained, and the average particle size of nonporous powders may be calculated.

Gas adsorption as a method of determining the surface areas of finely divided porous or nonporous solid materials is not new. The method has, however, only recently found real practical utility in measurements of absolute surface areas as a result of the theoretical developments projected by Brunauer, Emmett, and Teller (19) (BET Method). It has seen a particularly prolific application industrially to the determination of surface areas of catalysts. A number of chemically different adsorbate gases have been employed successfully to this end.

For the project being carried out in this laboratory, nitrogen was chosen as the adsorbate since liquid nitrogen could be conveniently prepared for the cooling bath. Likewise, since some of the adsorbents being studied are organic in nature, it seemed desirable to abstain from using organic gases which might react with or be dissolved in the organic crystals. The method, theory, and application of gas adsorption for this purpose have been the subject of a number of journal and review articles (20, 21, 22), so that the present paper shall treat this aspect only briefly.

APPARATUS AND PROCEDURE

Adsorption Apparatus.—The adsorption equipment was mounted on a metal framework. This was then built on a low table set on casters, permitting free portability. The apparatus was of a standard design, with a few modifications, as shown in Fig. 1.

The sizes of the adsorption tubes were dictated by the nature of the adsorbent, smaller tubes being used where the surface area per unit volume of adsorbent was high. Two-millimeter capillary tubing was used to connect critical portions of the system in order to keep dead-space volumes at a minimum yet allowing for a reasonably rapid evacuation of gases during evacuation procedures. In the powders reported in this paper, adsorption analyses were preceded by evacuation of the samples for ten hours at 105° in order to remove adsorbed water and other extraneous substances from the surface of the adsorbents. The relative vapor pressures of the adsorbent samples ultimately determined the temperatures used in this particular operation. With substances of relatively high vapor pressures at this evacuation temperature, the nature of the crystal surfaces would change appreciably by sublimation. The samples were evacuated at temperatures where the vapor pressure of the adsorbent remained well below 0.001 cm. of mercury. Space in the critical portion of the system, not occupied by either the sample or the calibrated burettes, was determined by employing pure helium since this gas is inert, is not adsorbed at room temperature, and obeys the laws of an ideal gas under the experimental conditions. This dead space, a, can be calculated by determining the pressure of a given amount of helium confined in the space plus variable known volumes, V', in the burettes. The equation would then take the form $P(V' + a) = \text{constant}$. The equation can be solved for a if several values of P are obtained for corresponding values of V'.

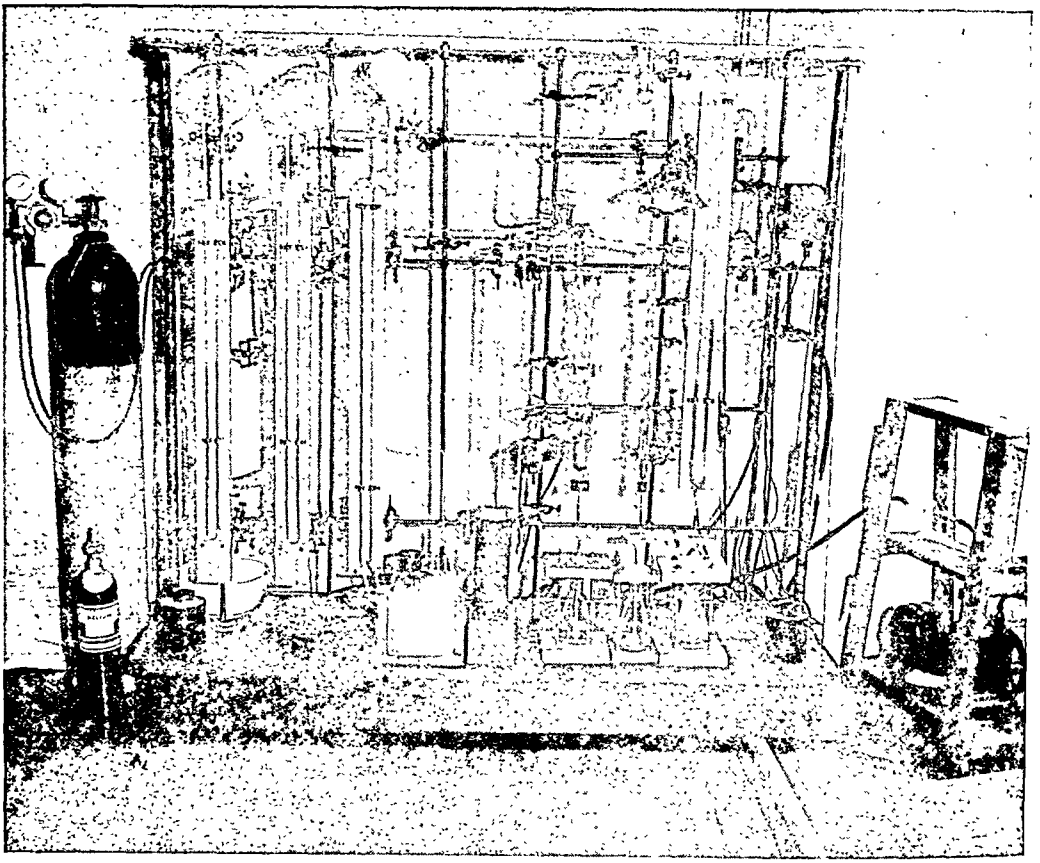

Fig. 1.—Adsorption equipment.

Since liquid nitrogen for cooling the sample tubes was not readily available, it was prepared by utilizing a liquid air condenser of simple design illustrated in Fig. 2.

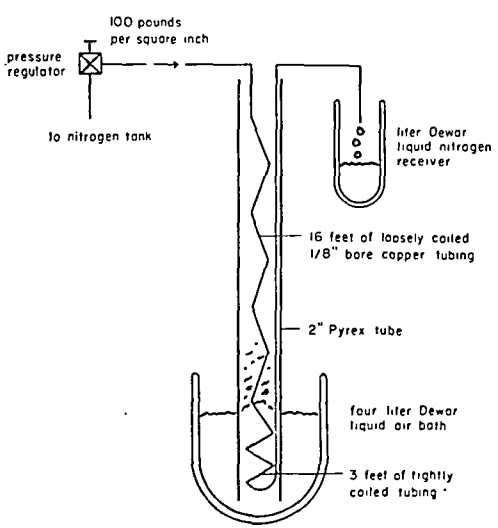

Fig. 2.—Schematic diagram of liquid nitrogen condenser.

Precooled gaseous nitrogen at a pressure of 100 lb. per square inch was passed through the liquid air immersed coils and a liter of liquid nitrogen was obtained for every three liters of liquid air consumed. Greater efficiency could be attained if quantities larger than one liter of nitrogen were prepared at a single operation.

The temperature of the liquid nitrogen bath varied only slightly during successive runs. The values of this temperature and P_0, obtained by employing a nitrogen vapor pressure thermometer, indicated that P_0 ranged consistently between 69 and 72 cm. of mercury and ranged usually between 70 and 71 cm. of mercury so that a value of 77° K. was used regularly as the liquid nitrogen bath temperature.[1]

[1] By calculations involving the Clausius-Clapeyron equation

$$\log \frac{P_1}{P_2} = \frac{L}{2.303R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where,

P_1 = 76 cm. of mercury; P_2 = value of P_0 observed on the vapor pressure thermometer; L = heat of vaporization of nitrogen, equal to 1323 calories per mole; R = the gas constant equal to 1.987 calories per degree per mole; T_1 = calculated absolute temperature for variable values of P_2; and T_2 = the equilibrium temperature of boiling liquid nitrogen in a nitrogen atmosphere at a pressure of 76 cm. of mercury, equal to 77.58° K.

It can be shown that T_1 is equal to 76.72° K. when P_0 (i.e., P_2 in the equation) is equal to 69 cm. of mercury and similarly T_1 is equal to 77.10° K. when P_0 is equal to 72 cm. of mercury. The deviation from an absolute value of 77° K. was thus so minimal that the latter value could be used consistently without introducing an error exceeding other experimental errors.

The adsorbate nitrogen used for these measurements was a specially pure grade of Linde tank gas which was used directly without further purification. The helium for dead-space measurements was a medical grade product supplied in cylinders by the Puritan Compressed Gas Corp. This gas was purified by conventional methods utilizing an activated charcoal train cooled to liquid nitrogen temperature.

Calculation.—The straight line isotherms obtained from the nitrogen adsorption data, were plotted in the relative pressure (P/P_0) range of 0.05 to 0.35 by substituting the appropriate experimental values in the *BET* equation

$$\frac{P}{V(P_0 - P)} = \frac{1}{V_m C} + \frac{(C-1)P}{V_m C P_0} \qquad (1)$$

where

V = volume of gas adsorbed per gram of adsorbent at pressure P at a temperature at which the vapor pressure of the liquefied gas is P_0,

V_m = volume of gas in cc. (converted to S. T. P.) required to form a monolayer on a gram of adsorbent,

C = a constant which is related exponentially to the difference between the heat of liquefaction and the heat of adsorption of the adsorbate.

From the slopes and intercepts, the respective values of V_m were obtained for the individual samples. The value of 16.2 Å.^{2} was used as the area occupied by a single close-packed nitrogen molecule adsorbed as a liquid monolayer film on the surface of the adsorbent. Thus one obtains the factor 4.35 which multiplied by the value of V_m yields directly the specific surface area in square meters per gram of adsorbent. In calculating the average particle size of the powders, the following equation was used:

$$d_m = \frac{6}{A\rho} \qquad (2)$$

where

d_m = surface average spherical diameter of particles in centimeters,

A = specific surface area in square centimeters of a gram of adsorbent, and

ρ = the density of the adsorbent material.

The specific surface areas of the following powders were determined in the apparatus described:

Titanium Dioxide.—The particular sample of titanium dioxide (anatase) studied was obtained from Professor W. D. Harkins of the University of Chicago. The material was from a lot which has been intensively studied by numerous investigators utilizing varied experimental approaches. For this sample Dr. Harkins obtained a value of 13.8 m^2/Gm. by using the energy of emersion technique. Several other workers employing the method of low-temperature nitrogen adsorption isotherms found an average value of 13.9 m^2/Gm.

Zinc Oxide.—Mallinckrodt U. S. P. grade zinc oxide was used without any pretreatment. The powder was dried over granular anhydrous magnesium perchlorate in a desiccator for at least a week, as were all other powders, prior to measurement.

Barium Sulfate.—Merck X-ray grade barium sulfate was used directly without further pretreatment aside from the drying operation.

RESULTS AND DISCUSSION

The results of the measurements on the three powders are shown in Figs. 3, 4, 5, and 6 and in

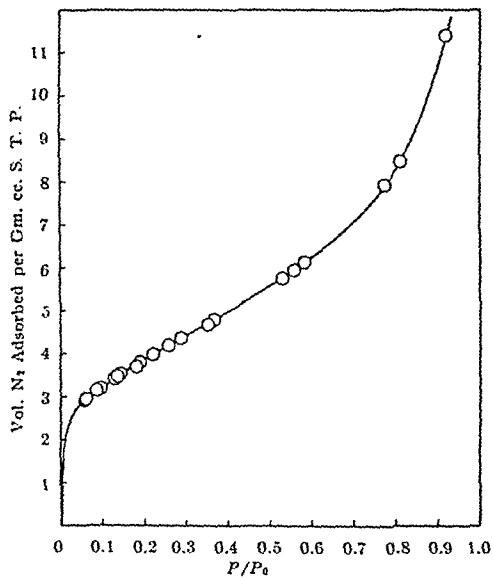

Fig. 3.—Adsorption of nitrogen on titanium dioxide (Sample No. 1A-6) at liquid nitrogen temperatures.

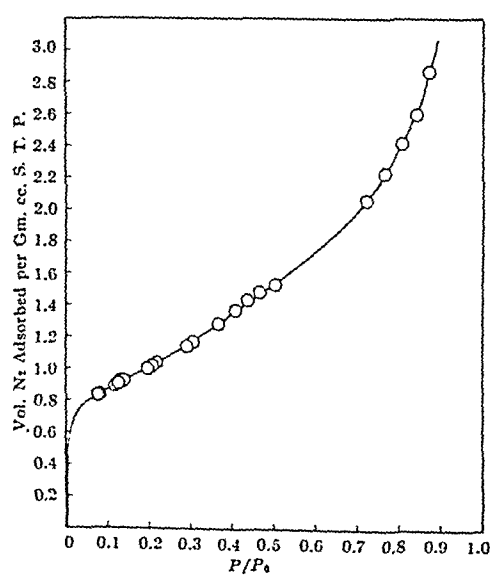

Fig. 4.—Adsorption of nitrogen on zinc oxide (Sample No. 2A-2) at liquid nitrogen temperatures.

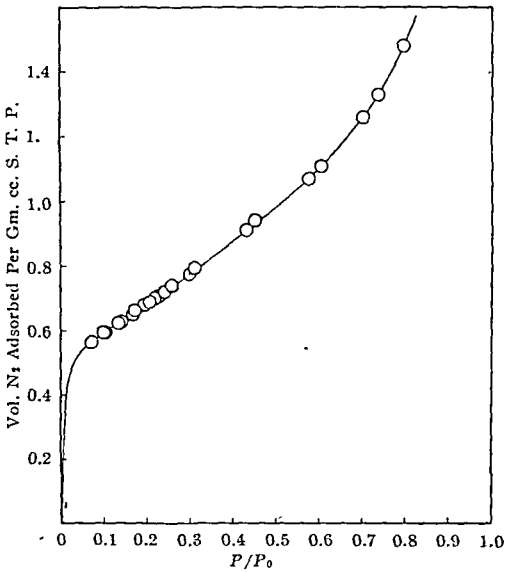

Fig. 5.—Adsorption of nitrogen on barium sulfate (Sample No. 3A-2) at liquid nitrogen temperatures.

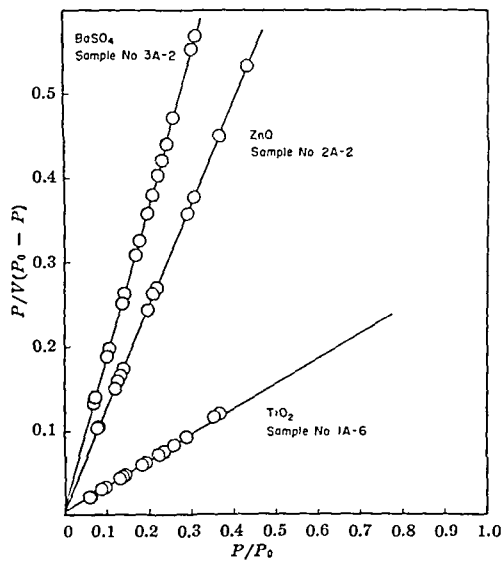

Fig. 6.—Adsorption data for nitrogen on titanium dioxide (Sample No. 1A-6), zinc oxide (Sample 2A-2), and barium sulfate (Sample No. 3A-2), plotted according to Eq. (1).

Table I. As shown in the tabulated data, numerous runs were made on the anatase sample in order to determine the relative and the absolute precision of the technique employed. The mean value of 13.7 m²/Gm. agrees satisfactorily with the results of other workers. For the sample of anatase, where ten separate values were obtained, the standard deviation of measurement calculated according to the equation

$$\sigma = \sqrt{\frac{\Sigma(x - x_0)^2}{n - 1}} \qquad (3)$$

TABLE I.—SURFACE AREA MEASUREMENTS ON SAMPLES OF TITANIUM DIOXIDE, ZINC OXIDE, AND BARIUM SULFATE BY NITROGEN ADSORPTION

Material	Sample Wt., Gm.	Analysis No.	V_m, cc.	Area, m²/Gm.
Titanium Dioxide[a] (Anatase), Sample No. 1A	2.247	1	3.20	13.9
	2.247	2	3.18	13.8
	3.530	3	3.26	14.2
	3.330	4	3.26	14.2
	3.310	5	3.07	13.4
	3.010	6	3.13	13.6
	3.000	7	3.10	13.5
	3.080	8	3.10	13.5
	3.150	9	3.10	13.5
	2.863	10	3.18	13.8[a]
Zinc Oxide,[b] Sample No. 2A	13.960	1	0.816	3.55
	4.050	2	0.816	3.55
	14.790	3	0.831	3.61
	13.087	4	0.811	3.52[b]
Barium Sulfate,[c] Sample No. 3A	20.086	1	0.549	2.39[c]
	17.060	2	0.557	2.42
	14.790	3	0.561	2.44

[a] Average area, 13.7 m²/Gm.; density, 3.84; Diameter of average particles, 0.114 μ.
[b] Average area, 3 56 m²/Gm.; density, 5.47; diameter of average particle, 0.308 μ.
[c] Average area, 2.42 m²/Gm.; density, 4.50; diameter of average particles, 0.551 μ.

is 0.29 m²/Gm. The coefficient of variation for the titanium dioxide sample therefore is about 2%. It would thus appear that the accuracy and reproducibility of the method are quite adequate for the type of investigations contemplated.

Though the samples of zinc oxide and barium sulfate do not show any detectable difference in size by visual examination or by feeling them, the measurements would indicate a considerable relative difference from the titanium dioxide with respect to the particle size. This would indicate that such qualitative visual comparisons break down completely in this particle size range.

Hysteresis was not observed in the measurements on any of these powders.

SIGNIFICANCE OF THE RESULTS

If the powders under investigation consisted entirely of perfectly uniform spheres or cubes, the diameters listed in the table would conform very closely with the actual dimensions of the particles. However, the assumption is obviously too optimistic and the actual particles are more probably represented by a spectrum of sizes, the range varying for different materials and conditioned somewhat by the method of manufacture. The determined particle sizes in the case of these powders would be a surface mean particle diameter. Mathematically this could be represented as

$$d_{\text{surface average}} = \frac{\Sigma d_i^3}{\Sigma d_i^2} \qquad (4)$$

where the summation is over all of the particles. In comparison, the other types of averages take the following forms:

$$d_{\text{number average}} = \frac{\Sigma d_i}{n} \quad (5)$$

where n = total number of particles and

$$d_{\text{weight average}} = \frac{\Sigma d_i^4}{\Sigma d_i^3} \quad (6)$$

It is evident from these relationships that the surface average values would be somewhere intermediate between those obtained from number and weight averages.

Though the particle diameters determined by the gas adsorption method lose some of their significance because of the nonuniformity in size of the powder particles, the specific surface area—the area of the solid-air interface per gram of powder—is a real, definite value. Since specific surface area measurements permit ready calculation of the total surface area present in any given amount of powder, it should prove interesting to see whether the effective doses of certain slightly soluble drugs can be more directly correlated with the surface areas than with the weights of these substances. The solution rates and consequently the adsorption rates should be directly related to the exposed surface areas and therefore to the measured specific surface areas.

SUMMARY

The application of low-temperature nitrogen adsorption isotherms to the determination of specific surface areas and surface average diameters of pharmaceutical powders is illustrated in data for titanium dioxide, zinc oxide, and barium sulfate. Representative samples of these substances gave specific surface area values of 13.7 m^2/Gm. for titanium dioxide, 3.56 m^2/Gm. for zinc oxide, and 2.42 m^2/Gm for barium sulfate. The possible correlations of specific surface areas of slightly soluble medicinal powders with solution rates, adsorption rates, and therapeutic response are pointed out.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Glucose monocarbonate (any isomer)
L-Galactonolactone
Carboxymethoxylamine hemihydrochloride
3,5-Diiodotyrosine-*p*-nitrobenzyl ether
Hydroxypyruvic acid
2-Dimethylamino-1-chloropropane
3,4-Dichlorophenyl isocyanate
Sylvestrine
3,5-Diiodothyronine
Chavicine
12-Hydroxystearic acid
18-Hydroxystearic acid
p-Menthane
Vanillyl amine
d-Pantoyltauryl-*p*-chloroanilide
Vaccenic acid
Thymidine
3-Hydroxyanthranilic acid
3,7,3′,4′,5′-Pentahydroxyflavone
3,5,7,2′-Tetrahydroxyflavone

Effects of Surface-Active Agents on the Crystallinity of Carbowax Compounds*

By M. V. NADKARNI,† DONALD B. MEYERS† and LOUIS C. ZOPF‡

"Carbowax" 4000, a water-soluble waxlike compound, is a useful ingredient in washable ointment bases. The texture, unctuousness, and smoothness of the "Carbowax" containing base depend upon the degree of fineness of the crystals of "Carbowax." Several factors affect the size and form of crystals. Presence of synthetic wetting agents and emulsifiers in comparatively minute amounts has been demonstrated to have considerable effect on the size of the crystals. Several ionic and nonionic wetting agents in various concentrations have been studied. Photomicrographs have been made from the slides depicting crystallization from various combinations. Preliminary experiments show that some of these wetting agents are efficient in reducing crystal size.

IN RECENT YEARS, there has been an increasing tendency on the part of physicians to employ washable ointment bases in preference to the traditional oleaginous vehicles. In view of the existing confusion in the proper use of the term "washable," it may be well to point out that, as used here, it refers to the ease with which these bases and resulting ointments can be removed from the skin and clothing with water.

A great majority of these washable bases contain the water-soluble "Carbowax" compounds.[1] During the progress of our experiments on hundreds of washable ointment bases containing "Carbowax," it was observed that occasionally a rough, grainy ointment base of undesirable appearance could be changed into a smooth, uniform product by incorporation of a very small amount of a wetting or emulsifying agent. For pharmaceutical elegance, an ointment base must possess certain physical properties among which smooth texture and unctuousness are of prime importance.

A comparative microscopic study of slides prepared from bases made with and without the addition of surface-active agents in the formula revealed that those containing these agents showed improved appearance and uniformity. There were indications that this was due to the reduction in the size of the "Carbowax" crystals brought about by the presence of the surface-active agents. A study of the crystal behavior of "Carbowax" compounds and the effects of various factors affecting the size and form of crystals was, therefore, considered pertinent.

Very little work has been reported so far regarding the features of crystal structure of water-soluble "Carbowax" compounds. Paraffin wax and other petroleum waxes, on the other hand, have been extensively investigated and numerous reports have appeared describing their crystal structure and factors affecting it (2, 3, 6, 7, 12, 13).

Several factors other than the presence of surface-active agents seem to have an effect on crystal size of "Carbowaxes." As with other crystallizable compounds, it was observed that the rapidity of formation of crystals, the temperature to which the wax had been heated before allowing it to cool and crystallize, the molecular weight of the wax, the rate of cooling of the melted wax, the degree of purity of the material, and the amount of agitation during cooling of the melted wax are a few of the factors influencing the size of the crystals.

During this study, special care was taken to maintain external factors as uniform as was practicable. Because of the necessity to work with minute amounts of the "Carbowax" in order to obtain slides for microscopic study, the difficulty of rigidly controlling external conditions may be appreciated. The same lot of "Carbowax" 4000 was employed throughout this study. The temperature to which the wax was heated initially was the same in all cases, and the rate of cooling of the melted wax to room temperature was maintained as uniform as possible by enclosing the slide in a box insulated with board asbestos. Details of the procedure are given later.

Surface-active agents investigated consisted both of the ionizable and the nonionic type. Water was included because it is a constituent of

* Received September 1, 1948, from the College of Pharmacy, State University of Iowa, Iowa City.
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‡ Professor of Pharmacy, College of Pharmacy, State University of Iowa.
[1] Products of the Carbide and Carbon Chemicals Corporation: They comprise a series of products of wax—like consistency having the general formula $HOCH_2—(CH_2—O—CH_2)n—CH_2OH$. (The number following the word "Carbowax" refers to the approximate molecular weight of the wax.) Their physical properties vary with their molecular weights. The outstanding features of "Carbowax" compounds are their solubility in water and ability to form unctuous products when mixed with certain diluents, e.g., water, glycols, glycerin, etc., which makes them suited for use as a water-soluble ointment base.

all hydrous washable bases. The following substances were studied:

(1) Water
(2) Tergitol 4. (*a*)
(3) Tergitol 4 T
(4) Tergitol 7
(5) Tergitol 08
(6) Tween 80 (*b*)
(7) Tween 60
(8) Duponol C (*c*)
(9) Span 65 (*d*)
(10) Span 85
(11) Span 80
(12) Span 60
(13) Span 40
(14) Aerosol AY (*e*)
(15) Polyethyleneglycol 400 monostearate (*f*)
(16) Polyethyleneglycol 400 distearate
(17) Polyethyleneglycol 600 distearate
(18) Polyethyleneglycol 1000 monostearate
(19) Polyethyleneglycol 1540 distearate

(*a*) Tergitols: Products of Carbide and Carbon Chemicals Corporation. These are sodium salts and amine salts of higher secondary alkyl sulfates.

Tergitol 4: $C_4H_9—\underset{\underset{C_2H_5}{/}}{CH}—C_2H_4—\underset{\underset{SO_4Na}{/}}{CH}—CH_2—CH—(CH_3)_2$

Tergitol 4T: Amine salt of higher secondary alkyl sulfate (Triethanolamine derivative of 7-ethyl-2-methyl-4-undecanol)

Tergitol 7: $C_4H_9—\underset{\underset{C_2H_5}{/}}{CH}—C_2H_4—\underset{\underset{SO_4Na}{/}}{CH}—C_2H_4—CH—(C_2H_5)_2$

Tergitol 08: $C_4H_9—\underset{\underset{C_2H_5}{/}}{CH}—CH_2—SO_4Na$

(*b*) Tweens: Atlas Powder Company
Tween 80: Polyoxyalkylene sorbitan monooleate
Tween 60: Polyoxyalkylene sorbitan monostearate

(*c*) Duponol C (Du Pont):
Sodium lauryl sulfate
$CH_3(CH_2)_{10}CH_2—O—SO_3—Na$

(*d*) Spans: Atlas Powder Company:
Span 85: Sorbitan trioleate
Span 80: Sorbitan monooleate
Span 60: Sorbitan monostearate
Span 40: Sorbitan monopalmitate

(*e*) Aerosol AY: Diamyl sodium sulfosuccinate (American Cyanamid Chemical Corporation)

(*f*) Polyethyleneglycol stearates: Carbide and Carbon Chemical Corporation and Kessler Chemical Company. These are mono- and distearates of the polyethylene glycols.

For observation of the crystal form of the pure waxes, a set of microscope slides were prepared. Another set was made to demonstrate the effects of addition of 10% to 40% of water to "Carbowax" 4000. The surface-active agents were employed in concentrations of 1% and 2 per cent. The aqueous phase of these respective mixtures was maintained at 10% and 20 per cent. The remainder of the mixture consisted of "Carbowax" 4000.

("Carbowax" 4000 was studied extensively because it has been demonstrated that, from the point of view of physical properties, "Carbowax" 4000 is the most suitable of the "Carbowaxes" so far examined for ointment formulation.)

PROCEDURE

"Carbowax" 4000 was heated to 70° in a small beaker on a water bath. The desired amount of the surface-active agent was weighed or measured and added to the molten wax with continuous stirring until a homogeneous mixture was obtained. A drop of the warm molten mixture at 70° was then removed with a small glass rod and carefully transferred to a microscope slide. A cover-slip was placed on the melted material without pressure and the slide immediately observed under the microscope.

Whenever water was employed in the formulation of the ointment base, the surface-active agent was separately dissolved in the water portion. This solution was heated to the same temperature as that of the wax before mixing. Further procedure was as above.

RESULTS

Observation of the gradual growth of "Carbowax" crystals under the microscope was an interesting and fascinating phenomenon. "Carbowax" compounds, without the addition of other ingredients, started crystallizing around a nucleus. Numerous dendrites radiated from this nucleus which was a random point. Similar growth from another nucleus coalesced with the first to form a ridge-like barrier. The crystals of pure "Carbowax" compounds were anisotropic; they showed a play of colors when observed under rotating Nicol's prisms. The crystals may be described generally as spherulitic plates.

SIZE OF CARBOWAX CRYSTALS

Wax	Average Size in mm.
Carbowax 1500	2.13 × 1.42
Carbowax 1540	1.2 × 0.78
Carbowax 4000	0.64 × 0.42
Carbowax 6000	0.28 × 0.21

Several slides were prepared for each concentration of the surface-active agents to insure that substantially the same type of crystals was formed each time under similar conditions.

Photomicrographs were made from each slide under polarized light since these gave a much improved definition and contrast in the final photographs (see pages 218 to 221). Both black and white and colored photographs were made. Magnification of the photomicrographs was the same in all cases, viz., about 400 diameters. The photographs are self explanatory with respect to the crystal size reduction. Only a few of the representative pictures are reproduced.

CONCLUSIONS

1. It appears that the physical properties of the waxes and their chemical constitution are an important factor in recrystallization, although

other associated substances mixed in deliberately or naturally also have considerable effect.

2. Upon slow cooling "Carbowax" compounds, *per se*, form large plate crystals from a nucleus. All members of the series show a marked similarity in crystal shape. They are described as plates with a nucleus and of spherulitic nature. The crystals display an anisotropic behavior revealed by a polarizing microscope.

3. The average size of the crystals formed from pure "Carbowaxes" under specified conditions diminishes as the molecular weight increases.

4. Addition of a diluent such as water reduces the average size of crystals but they still remain considerably larger than what may be termed desirable for an ointment base.

5. As a rule, crystals formed after the addition of surface-active agents lose their anisotropic nature and in some cases the form of the crystals changes from plate to the mal type. Mal type is characterized by lack of any definite recognizable form.

6. Surface-active agents have a definite effect on the size and form of "Carbowax" 4000 crystals. All those tested reduce the size, but their quantitative ability to reduce crystal size varies considerably.

7. The effects of the different surface-active agents and water may be briefly summarized:

(*a*) Water: Size of crystals is reduced but slightly by increasing the proportion of water. (Fig. 1-d.)

(*b*) Tergitols: Tergitol 4 in concentration of 1 per cent in presence of 9 per cent water reduces crystal size to a greater degree than any of the other surface-active agents studied. (Fig. 1-e.)

(*c*) Tweens: One per cent Tween 65 together with 9 per cent of water is superior to same concentration of Tween 80.

(*d*) Duponol C: Duponol is not very effective in reducing crystal size and does not give a uniform grain. (Fig. 1-g.)

(*e*) Spans: None of the spans are very effective in crystal size reduction. Plate-type crystals are changed to mal type (Figs. 1-h and i.)

(*f*) Aerosol AY is not greatly effective. (Fig. 1-j.)

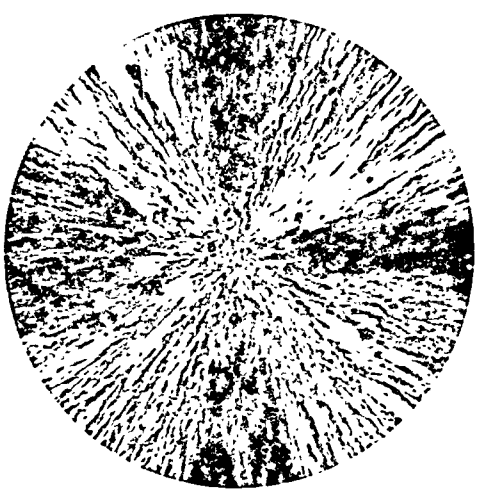
(*g*) Polyethylene Glycol Stearates: Polyethylene glycol 400 monostearate in concentration of 5 per cent and Polyethyleneglycol 400 distearate in concentrations of 5 and 10 per cent are effective crystal size reducers. Concentrations greater than 5 per cent of Polyethyleneglycol 400 monostearate in the former case and 10 per cent of Polyethyleneglycol 400 distearate showed no further reduction. (Figs. 1-l, m, o.)

(*h*) There appears to be an optimum concentration of any given surface-active agent which produces maximum effect on crystal size. Amounts in excess of this optimum are not only superfluous and without any further effect but sometimes have a deleterious effect on homogeneity of the product. (Figs. 1-l, m, n, o.)

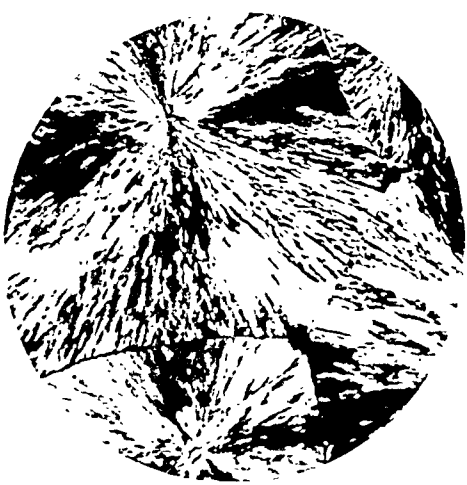
(*i*) Distearates of Polyethyleneglycols 600 and above do not seem to have any effect in reducing crystal size. (Figs. 1-p, q, r.)

(*j*) Whereas the composition of paraffin wax shows evidence of being complex and a mixture of different varieties of crystal forms (a-e, h-n, 8-14), evidence collected so far in the case of "Carbowax" compounds indicates that pure samples of "Carbowaxes" crystallize in single habit.

Fig. 1.—Photomicrographs showing the effect of various surface-active agents on the crystallinity of carbowax compounds.



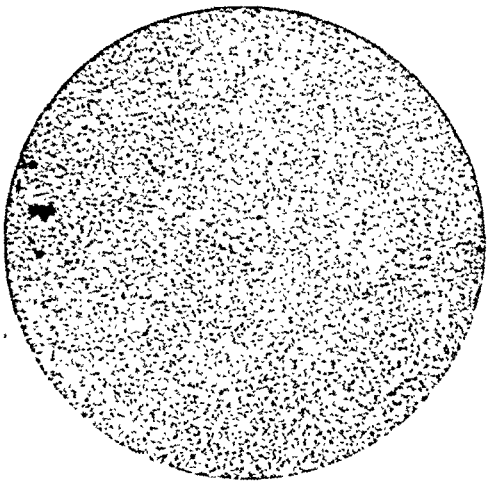
a. Carbowax 1540



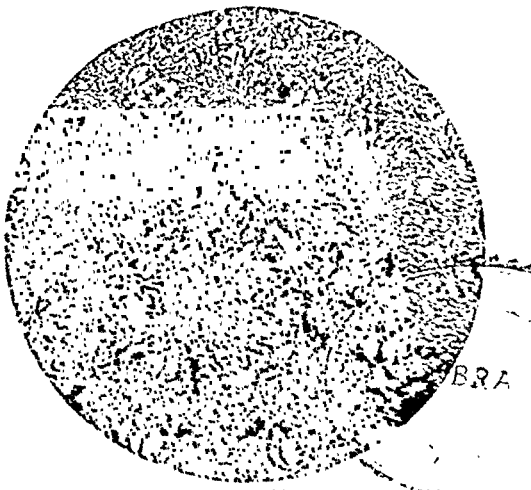
b. Carbowax 4000

c. Carbowax 6000

d. Carbowax 4000 with 10% water

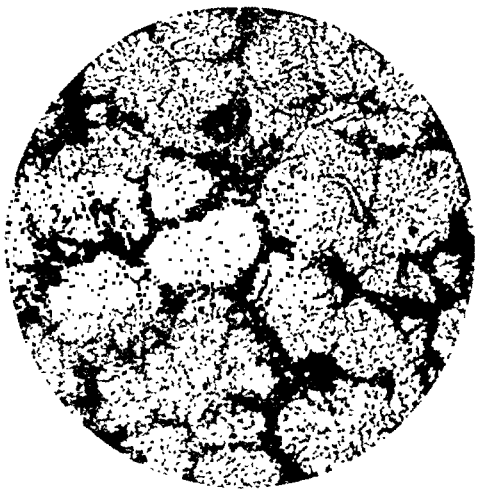


e. Carbowax 4000 with 10% water and 1% Tergitol 04



f. Carbowax 4000 with 10% water and 1% Tergitol 08

g. Carbowax 4000 with 10% water and 1% Duponol C

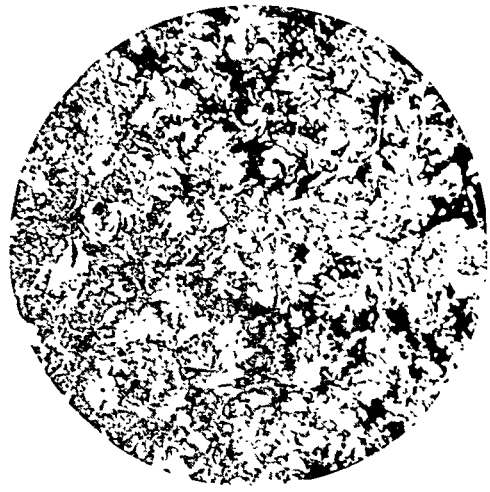


h. Carbowax 4000 with 10% water and 1% Span 40

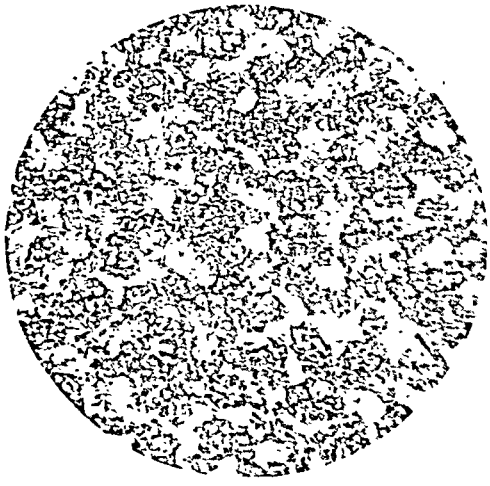
Fig. 1 (*Continued on page 220*)

Fig. 1 (*Continued from page 219*)

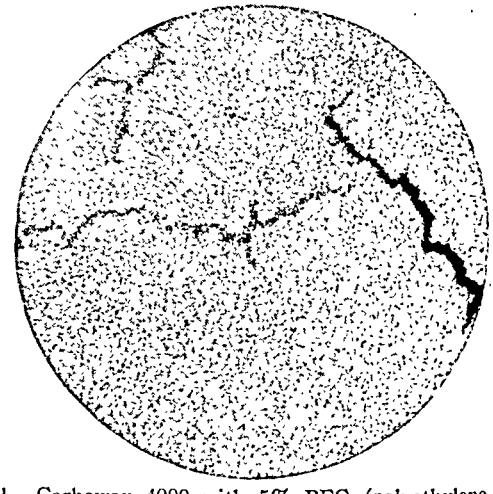
i. Carbowax 4000 with 10% water and 1% Span 60



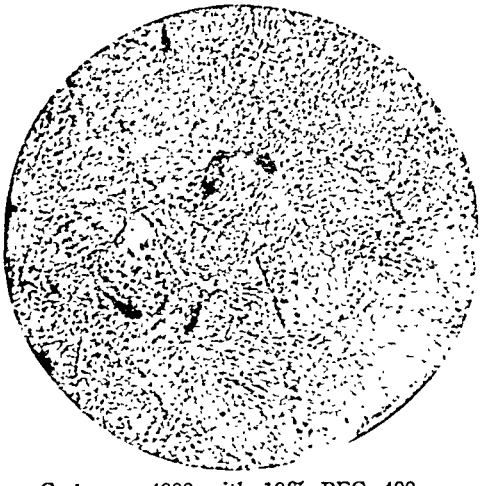
j. Carbowax 4000 with 20% water and 2% Aerosol AY



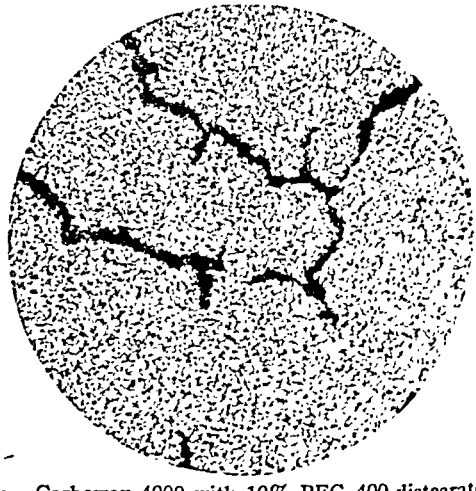
k. Polyethyleneglycol 400 monostearate



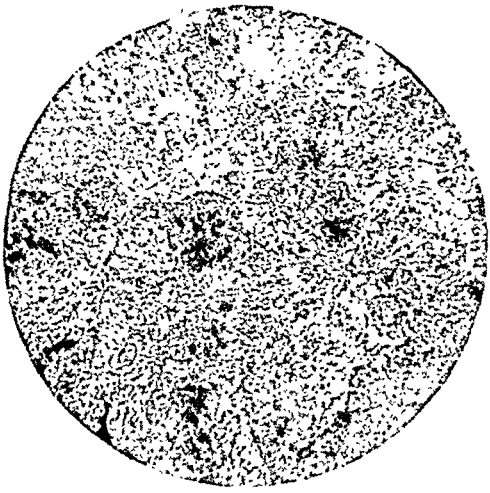
l. Carbowax 4000 with 5% PEG (polyethyleneglycol) 400 monostearate



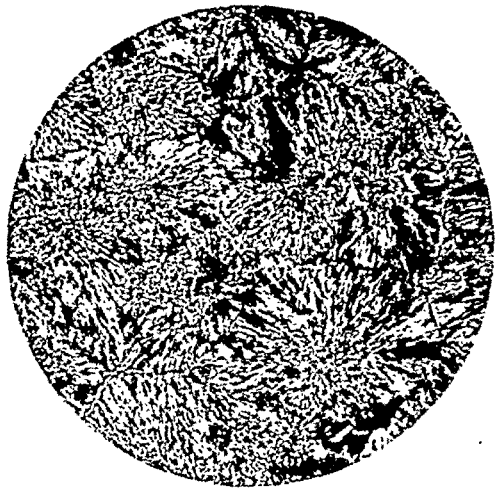
m. Carbowax 4000 with 10% PEG 400 monostearate



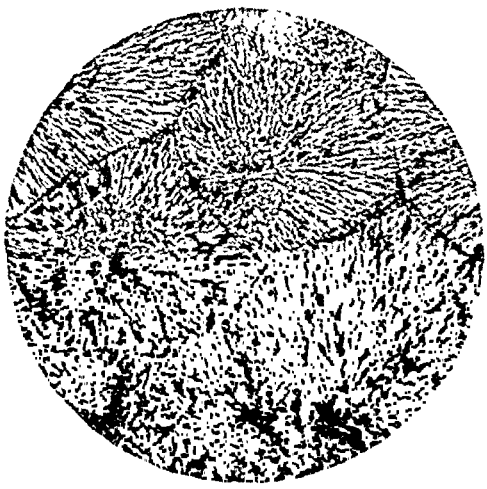
n. Carbowax 4000 with 10% PEG 400 distearate



o. Carbowax 4000 with 20% PEG 400 distearate



p. Carbowax 4000 with 10% PEG 600 distearate



q. Carbowax 4000 with 10% Carbowax 1000 monostearate

r. Carbowax 4000 with 10% Carbowax 1540 distearate

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A Critical Study of the Diffusion of Iodine and Sulfathiazole from Ointments*,†

By LAURENCE D. LOCKIE‡ and JOSEPH B. SPROWLS§

Present *in vitro* methods of the study of diffusion of drugs from ointments into gels or solutions are essentially studies of diffusion rates. A physico-chemical approach to the study of diffusion rates is described in this paper. Iodine and sulfathiazole were incorporated into various ointment bases. Types of bases included greases, water-in-oil emulsions, and mucilages. The diffusion into agar gels containing appropriate reagents was recorded at definite time intervals. Diffusion rates, when plotted on graph paper, were found to follow a general pattern, when oil-in-water emulsion and mucilage bases were used.

In recent years, the subject of diffusion from ointment bases has received much attention from both medical and pharmaceutical investigators, particularly with regard to the relative merits of various types of ointment bases as carriers of the newer types of therapeutic agents.

Methods of study have been carried on along two general lines—*in vitro* methods and those involving clinical data. While the final test of the value of any therapeutic agent is its clinical performance, the *in vitro* methods of study are most interesting to the theoretical pharmacist, since they lend themselves to more accurate study and delineation than do the clinical methods. Furthermore, while the *in vitro* methods which have been developed do not closely parallel the actual conditions of use, experience has shown that, for those drugs which are utilized for a purely local effect (for example, the sulfonamide drugs as local disinfectants), the *in vitro* studies have led to the same general conclusions as those which have been reached in the clinical studies.

This has been extensively documented in a recent publication by Huyck, *et al.* (1), in which it was shown that a large majority of the investigators using clinical methods prefer the oil-in-water emulsion type of ointment for the local application of sulfonamides, except in the treatment of burns. *In vitro* tests have indicated that the oil-in-water emulsion type of ointment base releases sulfonamides most readily (1–4).

In vitro studies have primarily been conducted by the agar plate method which was developed by Reddish (5) and described by Ruehle and Brewer (6). This method depends upon the zone of inhibition of bacterial growth which is produced when a given ointment is placed in the center of a Petri dish containing agar which has been uniformly seeded with a bacterial culture. The width of the clear zone (indicating inhibition of bacterial growth) is an indication of the facility of the ointment base to release its content of germicidal agent. This method has gained considerable prominence because of its former acceptance by the Food and Drug Administration as a recognized method for the testing of ointments and other types of germicidal preparations. Other investigators have studied by colorimetric and other methods the diffusion of drugs from ointment bases into agar gels, saline solution, blood serum, and water.

OTHER METHODS

The method developed by Waud and Ramsay (3) and later used by Huyck, *et al.* (*loc. cit*), was as follows: "A 4% solution of agar in Ringer's solution was prepared, and to this was added Ehrlich's solution, which in contact with sulfonamides turns red. After being placed in test tubes and allowed to gel, a layer of the vehicle containing the sulfonamide was placed over the gel, and the extent of the diffusion measured at definite intervals."

Bandelin and Kemp (2) measured the diffusion into saline solution and serum by the following method: "Four grams of the ointment was applied evenly to the inside surface of a 6- x 1-inch test tube, using a glass rod to spread the ointment in a uniform layer with a circular motion. Twenty-five cubic centimeters of saline or serum were then pipetted into the tube (capacity 60 cc.) and the tubes then placed in an incubator at 37.5° for varying lengths of time. Five tubes were thus prepared for each ointment, and one taken for analysis after two, four, six, eight, and twenty-four hours' incubation. The contents of the tube taken for analysis were poured carefully into another tube and agitated by inverting three times. An aliquot of this, taken for analysis, was used directly, or after appropriate dilution, depending upon the relative strength of the drug in the liquid. The amount of sulfonamide in the aliquot was determined colorimetrically by the method of Bratton and Marshall (7), using an electrophotometer and a filter with a maximum transmission at 525 mμ." They also checked their results by means of

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the F. D. A. agar cup plate method, a modification of the agar plate method previously described, wherein 1.5-cm. cups are cut in the agar layer in the Petri dish, and the ointment to be tested is placed within the cup.

R. W. Howard (4) spread the ointment to be tested on a watch glass and placed this in a covered jar containing 100 cc. of water. The jar was incubated at 37.5°. Then, at intervals of six, twelve, twenty-four, forty-eight, and ninety-six hours, 1-cc. samples were withdrawn and assayed by the Bratton and Marshall method.

It is the authors' thesis that since both of these general methods are based upon the diffusion of drugs into gels or solutions, the study of ointment bases by *in vitro* methods is essentially a study of diffusion rates. We therefore believe that a physicochemical approach to the study of ointment bases would be of value. Since the germicidal or therapeutic value of the drug itself can be evaluated by separate means, a measurement of the ability of an ointment base to release its drug content would seem to be an important indication of its potential value.

This statement, of course, gives no consideration to the emollient or protective value which a base might possess of itself. This is often of considerable importance, sometimes sufficiently so as to overshadow other considerations. In this study we are concerning ourselves with those instances in which the release of active ingredients from the ointment base is of primary importance.

EXPERIMENTAL

The drugs selected for these tests were iodine and sulfathiazole, since test solutions which show a color change and can be incorporated in an agar gel are readily available. Ehrlich's paradimethylaminobenzaldehyde solution gives a bright yellow color with sulfathiazole, and starch was used to show the penetration of the iodine. Sulfathiazole was used in the ointments rather than the sodium salt, since Waud and Ramsay (3) have observed that the diffusion of the sodium salt is only slightly faster than that of the sulfathiazole itself.

In preparing the iodine ointments, a solution of iodine was prepared, using 100 Gm. of iodine, 100 Gm. of potassium iodide, and distilled water to make 200 cc. The proper amount of the solution to obtain the desired amount of iodine in the base was then incorporated in the base.

Ointment bases were selected from the following types: (*a*) grease bases, (*b*) water-in-oil emulsion bases, (*c*) oil-in-water emulsion bases, and (*d*) mucilage bases.

A larger proportion of oil-in-water emulsion bases was included since the formulas for these differ widely, and the previous investigators have shown that diffusion from the other types is limited. Some of each, however, were included for purposes of comparison. A further comparison was provided for by using an aqueous solution containing the same concentration of drug as was contained in the ointments. Sodium sulfathiazole was used in the aqueous solution.

Following is a list of the bases used:

GREASE BASES

Petrolatum, U. S. P. XIII (hydrocarbon base)
Wool Fat, U. S. P. XIII (natural fat containing cholesterol)
Crisco (hydrogenated vegetable oil)
Sulfated Hydrogenated Castor Oil (water-miscible grease)

WATER-IN-OIL EMULSION BASES

Hydrophilic Petrolatum, U. S. P. XIII, with 30% water added
Lanolin, U. S. P. XIII

OIL-IN-WATER EMULSION BASES

Hydrophilic Ointment, U. S. P. XIII
Beeler's Base
Schwartz Base
Vanishing Cream "A"
Vanishing Cream "B"
Anhydrous Chipped Ointment Base (Rare Chemicals)
Carbowax-Petrolatum Base
Aerosol OT Emulsion
Diglycol Stearate Emulsion
Sulfated Hydrogenated Castor Oil (SHCO) Emulsion

The oil-in-water emulsion bases were selected to represent different emulsifiers and also to include formulas which had been suggested by researchers in the field. Hydrophilic Ointment, Beeler's Base, and the Schwartz Base all contain sodium lauryl sulfate; but in the first two the balance of the formula differs, and in the Schwartz formula no water is included. The Vanishing Creams "A" an "B" were taken from the Third Edition of the Pharmaceutical Recipe Book. "A" is a formula containing triethanolamine as the emulsifier, and "B" is a formula using potassium stearate. The Aerosol OT formula was included because it represented a truly sulfonated compound, and the SHCO emulsion to include a sulfated emulsifier. Rare Chemicals Anhydrous Chipped Ointment Base is a mixture of cetyl alcohol, stearyl alcohol, and a polyalkylene derivative of stearic acid.

MUCILAGE BASE

Tragacanth Paste.

This is the tragacanth paste formula from the Third Edition of the Pharmaceutical Recipe Book.

References for the other formulas follow: Beeler's Base, J. AM. PHARM. ASSOC., Practical Edition, 3, 231(1942); Schwartz Base, *ibid.*, 4, 111(1943); Aerosol OT Emulsion, J. AM. PHARM. ASSOC., Scientific Edition, 35, 129–140(1946); SHCO Emulsion, *ibid.*, 30, 145(1941); and Diglycol Stearate Emulsion, "Glyco Cosmetic Manual," Glyco Products Co., 148 Lafayette St., New York City, p. 62.

Formulas not available from the literature are:

RARE CHEMICALS OINTMENT BASE

Anhydrous Chipped Ointment Base[1]	30.0 Gm.
Distilled Water.....	70.0 cc.

[1] Rare Chemicals Anhydrous Chipped Ointment Base is a mixture of cetyl alcohol, stearyl alcohol, and a polyalkylene derivative of stearic acid.

Melt the base to 80°, heat the water to the same temperature. Add the water to the base with stirring. Stir until cool.—Rare Chemicals, Inc., Harrison, N. J.

CARBOWAX PETROLATUM BASE

Carbowax 1500	20.0 Gm.
Heavy Liquid Petrolatum	10.0 Gm.
Petrolatum	50.0 Gm.

Melt liquid petrolatum and petrolatum together, add carbowax and stir until cool.—Carbide and Carbon Chemicals Corp., 30 E. 42nd St., New York, N. Y.

PROCEDURE

A 4% agar gel was prepared, with the suitable reagent, and poured into standard 6-in. test tubes. When cool, the ointment bases containing the desired strengths of the drug to be tested were superimposed upon the gel in a layer approximately 3 cm. deep. The diffusion was then measured at appropriate time intervals. Since it was desirable to establish the character of the early portion of the curve, several readings were taken during the first day.

AGAR GELS USED

1. For Sulfathiazole

Bacto-agar (Difco)	4.0 Gm.
Ehrlich's Solution	1.0 cc.
Ringer's Solution, U. S. P. XIII	100.0 cc.

The agar was added to the boiling Ringer's Solution and stirred gently until uniform. The Ehrlich solution was added just before congelation and mixed thoroughly.

2. For Iodine

Bacto-agar (Difco)	4.0 Gm.
Starch (Soluble)	0.1 Gm.
Ringer's Solution, U. S. P. XIII	100.0 cc.

The agar and the starch were added to the boiling Ringer's solution and stirred gently until uniform.

The results of the first series of tests conducted are shown in Tables I and II.

DISCUSSION OF RESULTS

The sulfathiazole showed almost no diffusion from grease or water-in-oil emulsion bases. The rate of diffusion was fairly uniform from the oil-in-water emulsion bases and the mucilage base, although the rate from the SHCO emulsion was quite low. This low rate may have been due to experimental error, although in all cases at least two samples were tested, and if the results were not in reasonable agreement, the tests were repeated.

Iodine showed a much higher diffusion rate than did the sulfathiazole from both the oil-in-water emulsion bases and the solution. In this case the carbowax base showed a higher diffusion rate than the other bases, but not as high as the aqueous solution. Some diffusion was also observed from the

TABLE I.—DIFFUSION FROM 10% SULFATHIAZOLE OINTMENTS

Base \ Time in Hours:	1	3	6	24	48	72	96	120	168
	Distance in Millimeters								
Petrolatum	T[a]	T	T	5	5	T	T	..	T
Wool Fat	T	T	T	T	T	T	T	..	T
Hydrogenated Vegetable Oil	T	2	3	T	T	T	T	..	T
SHCO	1	2	3	T	T	T	T	..	T
Hydrophilic Petrolatum (30% Water)	T	T	T	5	5	5	T	..	T
Lanolin	0	T	T	T	T	T	T	..	T
SHCO Emulsion	2	3	4	10	12	14	15	15	..
Schwartz Base	2	4	6	11	20	25	27	30	..
Carbowax Base	5	8	10	18	25	31	35	..	43
Hydrophilic Ointment	4	6	8	14	24	28	30	..	40
Vanishing Cream "A"	4	5	7	15	24	28	30	36	40
Diglycol Stearate	3	5	7	15	25	29	33	39	..
Rare Chemicals Base	3	4	6	11	21	24	28	30	..
Beeler's Base	3	5	7	14	26	29	31	38	..
Aerosol OT Base	3	5	7	14	28	31	36	42	..
Vanishing Cream "B"	4	5	7	12	25	29	31	38	..
Tragacanth Paste	4	5	8	15	23	26	30	..	40
10% Aqueous Solution	5	7	10	21	33	39	43	..	..

[a] T means trace.

TABLE II.—DIFFUSION FROM 5% IODINE OINTMENTS

Base \ Time in Hours:	1	3	6	24	48	72	96	120	168
	Distance in Millimeters								
Wool Fat	0	2	2	3	5	6	6	6	7
Hydrophilic Petrolatum (30% Water)	2	4	5	7	9	10	10	11	11
Hydrophilic Ointment	6	10	13	26	38	45	53	59	69
Vanishing Cream "A"	6	11	14	27	36	44	52	58	70
Tragacanth Paste	7	11	14	27	40	48	56	63	74
Carbowax Base	7	10	17	28	43	55	68	80	End of tube
5% Aqueous Solution	8	13	19	39	55	65	77	86	End of tube

grease bases and the water-in-oil emulsion bases, although it was not large.

When the results shown in Table I were plotted on graph paper, with the distances in millimeters as ordinates, and the time in hours as abscissae, the curves showed the same resemblance to parabolic curves as did those recorded by other investigators.

A study of these curves showed that the points observed at each time interval for the oil-in-water emulsion base ointments vary somewhat, but not greatly. The curves showing maximum and minimum diffusion at each time interval are shown in Figure 1. This presented the possibility that experimental error might account for the variations. It was therefore decided to average all the data for each time interval except those for the SHCO emulsion and the aqueous solution. These average figures were then plotted on graphs, and gave the curves shown in Fig. 2 for the sulfathiazole ointments, and in Fig. 3 for the iodine ointments.

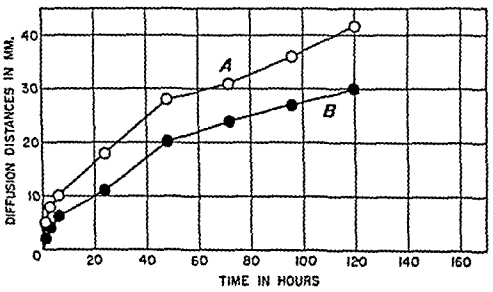

Fig. 1.—Variation in diffusion distances at each time interval.

Data from Table I, with diffusion distances in millimeters as ordinates, and times in hours as abscissae. 10% sulfathiazole ointments.

Curve A. Greatest distance.
Curve B. Smallest distance.

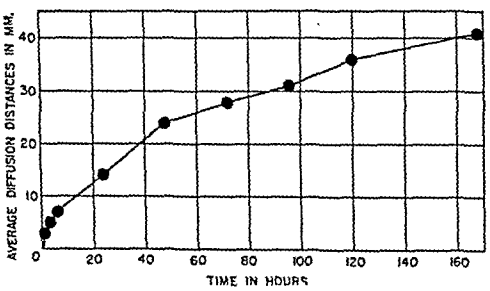

Fig. 2.—Average curve for 10% sulfathiazole ointments.

Diffusion curve for oil-in-water emulsion base ointments with 10% sulfathiazole. Ordinates are average figures for each time interval. Abscissae are times in hours.

Hours	Distance, Mm.	Hours	Distance, Mm.
1	3	72	28
3	5	96	31
6	7	120	36
24	14	168	41
48	24		

Since these were empiric curves, it was decided that a mathematical approach to the problem would be more accurate. Curves of this type are expressed by equations of the type $y^2 = kx$, where the curve passes through the origin. It was assumed for the moment that the curve did pass through the origin, i.e., that the "interface" between the ointment and the agar gel had no effect upon the diffusion time.

Because the rate of change of the slope (coefficient of x, k in the above equation) is not readily determined for curves of this type, advantage was taken of the fact that these curves may be converted to straight lines by setting y^2 equal to another variable, such as z, whereupon the equation becomes $z = kx$ (the equation of the straight line) (8). The slope of this equation then may be readily determined from experimental data by the method of averages (8). This was done in the case of the aqueous solutions of both iodine and sulfathiazole, and for the average data in the cases of the oil-in-water

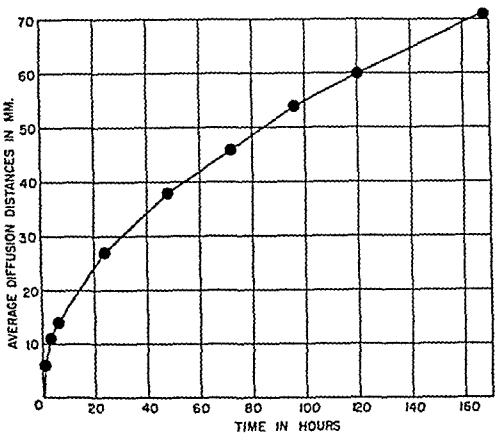

Fig. 3.—Average curve for 5% iodine ointments.

Diffusion curve for oil-in-water emulsion base ointments with 5% iodine. Ordinates are average figures for each time interval. Abscissae are times in hours.

Hours	Distance, Mm.	Hours	Distance, Mm.
1	6	72	46
3	11	96	54
6	14	120	60
24	27	168	71
48	38		

emulsion base ointments of both drugs. The coefficient of x was then rounded off to a convenient number which was well within the probable limit of error with the following results:

Aqueous Iodine Solution.....	60 ($z = 60x$)
Iodine Ointments.....	30 ($z = 30x$)
Aqueous Sulfathiazole Solution.	20 ($z = 20x$)
Sulfathiazole Ointments.....	10 ($z = 10x$)

To further illustrate, the line representing the equation $y^2 = 10x$ was drawn, as in Fig. 4, and the data for 10% sulfathiazole oil-in-water emulsion base ointments plotted to show their relationship to the mathematically determined curve.

Since the coefficient of x expresses the slope of curves of this type, and hence the diffusion rate, it would seem that this number might well be used to express the diffusibility of a substance from a base. Therefore, the coefficient for each oil-in-water emulsion base (and others where it was possible) was determined as above. These coefficients are recorded in Table III.

From Table III the following observations are drawn: The variations from the coefficient for all the oil-in-water emulsion base ointments with sulfathiazole, which was 10.36, are not large enough to prevent showing that they follow a generally similar pattern. The gap between these coefficients and the coefficient of the aqueous solution is much greater than those between the individual coefficients. The coefficients for the carbowax base (13.3) and the Aerosol emulsion (13.3) are high, and the coefficients for the Schwartz Base (7.7) and the Rare Chemicals Base (7.9) are low. Bandelin and Kemp (2) experienced variations in their determinations of about the same magnitude.

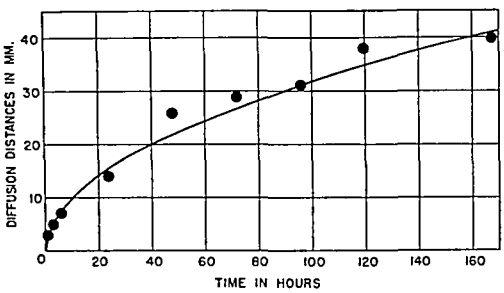

Fig. 4.—Mathematically determined curve.
Curve for the equation $y^2 = 10x$, with points for the diffusion of a 10% sulfathiazole ointment from an oil-in-water emulsion base (Beeler's), shown for purposes of comparison.

DATA

Ordinates, Diffusion in Millimeters	Abscissae, Time in Hours	Ordinates, Diffusion in Millimeters	Abscissae, Time in Hours
3	1	26	48
5	3	29	72
7	6	31	96
14	24	38	120

The general similarity of results obtained with various bases of the same type, and the reproducibility of data indicate that further studies by this method may lead to information from which generalities might be drawn.

For example, the average coefficient of x for 5% iodine in oil-in-water emulsion bases is about 30, while in water solution it is about 60. Also, the average coefficient for sulfathiazole in oil-in-water emulsion bases is about 10, while that for the 10% aqueous solution is about 20. In both cases, the ratio of solution coefficient to oil-in-water emulsion coefficient is 2:1. If further investigation were to indicate that the same relationship holds for other substances, it is possible that the diffusion rate for oil-in-water emulsion bases may be determined by experimental work on the solution.

In the same manner, the relationship between the diffusibility of iodine and sulfathiazole is noted to be 3:1. If such a relationship could be demonstrated to be generally true, then by setting up some substance as a standard, the diffusibility of other substances could be expressed by comparison with this standard. However, much more work must be done before general conclusions such as these can be presented.

BACTERIOLOGICAL TESTS

As a check on the validity of the test-tube method some tests were made by the agar plate method previously described (2). A layer of agar gel, uniformly seeded with *Staphylococcus aureus*, was placed in a Petri dish. One gram of the ointment was placed in the center of the dish. After incubation at 37.5° for twenty-four hours the average width of the clear zone was measured. The results are shown in Table IV where they are compared with the coefficient of x of the bases used.

TABLE III.—COEFFICENT OF x FOR VARIOUS OINTMENT BASES AND SUBSTANCES

Base	Coefficient of x
10% Sulfathiazole Ointments	
SHCO Emulsion	2.8
Schwartz Base	7.7
Carbowax Base	13.3
Hydrophilic Ointment	10.3
Vanishing Cream "A"	10.3
Diglycol Stearate Emulsion	11.4
Rare Chemicals Ointment Base	7.9
Beeler's Base	11.0
Aerosol OT Emulsion	13.3
Vanishing Cream "B"	10.6
Tragacanth Paste	9.8
10% Aqueous Solution	21.2
5% Iodine Ointments	
Wool Fat	0.46
Hydrophilic Petrolatum (30% Water)	1.5
Hydrophilic Ointment	29.0
Vanishing Cream "A"	28.0
Tragacanth Paste	32.4
Carbowax Base	42.8
5% Aqueous Solution	61.2

TABLE IV.—AGAR PLATE TESTS ON OINTMENTS

Base Used	Average Width of Clear Zone, Mm.	Coefficient of x
A. 2% Iodine		
Wool Fat	0.6	0.46
Petrolatum	4.5	...
SHCO	17.0	...
Schwartz Base	27.5	3.1
SHCO Emulsion	35.0	2.3
Diglycol Stearate	50.0	9.4
B. 10% Sulfathiazole		
Wool Fat	Trace	...
Petrolatum	Trace	...
SHCO	4.0	...
Schwartz Base	10.0 partial, a few colonies in zone	7.7
SHCO Emulsion	15.0	2.8
Diglycol Stearate	25.0	11.4

The results of these tests, as in the case of Bandelin and Kemp (2), were in general accordance with the results obtained by the test-tube method. Grease bases showed very small zones of inhibition, while the zones of inhibition were largest in the case of the oil-in-water emulsion bases. The iodine ointments also showed much larger zones than the corresponding zones for the sulfathiazole ointments, although the strength of the iodine ointments was reduced to 2%.

SUMMARY

From the results of the series of tests conducted, the following conclusions may be reached:

1. The diffusion rates of drugs from ointments may be expressed in parabolic curves of the form $y^2 = kx$, in which y represents the diffusion distance in millimeters, and x the diffusion time in hours.

2. These curves may also be expressed in terms of linear equations of the form $z = kx$, in which z represents y^2 of the previous equation.

3. The coefficient of x (k in the foregoing equations) may be used to express the diffusion rate of a substance at a given strength from a given base.

4. The method described seems well adapted to critical studies from which rather broad conclusions may be reached.

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The Antidiuretic Activity of the Posterior Pituitary and Its Quantitative Evaluation*

By K. M. LINDQUIST and L. W. ROWE

Using the method of Edmunds and Cushny as recently modified by Fugo and Aragon (rabbits intravenously), a total of 134 animals, made diuretic by glucose given intravenously, were tested with "Pitressin" and other posterior pituitary fractions to show quantitatively their antidiuretic activity. In most cases the relative antidiuretic activity was in good agreement with the relative pressor values but notably the International Reference Standard was only about 75 per cent as active as the U. S. P. Reference Standard instead of being equally active. One or two modifications are suggested which seem to make the method more accurate than the white mouse and rat methods previously used for antidiuretic assays.

THE METHOD of Burn (1) utilizing white rats injected subcutaneously has been widely used to determine the antidiuretic activity of definitely active posterior pituitary preparations. Considerable personal experience with this method has shown that it is time-consuming and not very accurate unless crossover comparisons are made between standard and unknown with the same groups of rats which makes it still slower in arriving at a result.

The mouse method of Gibbs (2) as modified by Grote, Jones, and Kamm (3) has been used in our laboratories with considerable success but it is also slow and somewhat inaccurate.

EXPERIMENTAL

Recently Fugo and Aragon (4) have suggested a modification of the technique of Edmunds and Cushny (5) which seemed to be both rapid and sensitive. This method utilizes small to medium-sized rabbits which have been given 3 mg. per Kg. of morphine sulfate subcutaneously and 1.5 Gm. per Kg. of urethane intraperitoneally. The jugular vein is then cannulated for infusion of warm saline and the bladder or the urethra is cannulated for collection of the urine. One modification suggested (4) was to give water by stomach tube (5% of the rabbit's body weight) three hours before the actual start of the antidiuretic experiment. We did this for about one-sixth of our 134 experiments. For the next two-thirds we injected the water directly into the stomach of the anesthetized rabbit through a long needle attached by rubber tubing to a 50-cc. burette. The last 20 of our experimental animals received no water orally since it seemed generally to interfere with the uniformity of urine excretion and also delayed the start of the experiment so that fewer comparative tests could be made on the same animal. Another modification (4) is the injection intravenously of 20 to 40 cc. of 20% glucose solution to

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start diuresis. This we found valuable and we standardized on a single injection of 25 cc. of the 20% glucose given intravenously thirty minutes before the first saline infusion of 50 cc. Urine was then collected for forty-five minutes and measured at five-minute intervals, immediately after which the dose of posterior pituitary was injected intravenously and the urine again collected for forty-five minutes. This permitted several injections of sample and standard for comparison in the course of an eight-hour working day.

A typical protocol is given in detail at the top of page 230.

Table I gives the results at a standard "Pitressin" dose versus an unknown dilution.

Table II gives the comparative pressor and antidiuretic activities of 16 different posterior pituitary preparations. The first eight and the last of the samples in the table are regular "Pitressins" or "Pitressin" fractions while the other seven were "Pitressin" Tannate samples that were made soluble with very small amounts of 50% acetic acid and then highly diluted before use. In general the agreement between pressor and antidiuretic activities was very good but it seemed to be a little better in the case of the tannate samples than with the others. Of course this rabbit method tests only the total antidiuretic activity of the tannate samples and tells nothing about their relative repository action just as the dog method gives only their total pressor activity.

Table III gives the comparative results obtained on 21 rabbits between the U. S. P. and the International Reference Standards as well as between the U. S. P. Standard and the standard "Pitressin." The last two appear to be about equal in antidiuretic activity but, except on rabbits Nos. 63, and 66, the International Standard seemed appreciably less active than the U. S. P. Standard. This was quite unexplainable since their antidiuretic activity had been found to be practically equal by the Burn rat method in collaborative tests of the old and new.

TABLE I.—STANDARD "PITRESSIN" (20 PRESSOR UNITS PER CC.) VERSUS AN UNKNOWN DILUTION

Rabbit No.	Wt., Kg	Cc. Urine, Before	Dose, Unit	Product	Cc. Urine, After	Antidiuretic Effect, %
3	1 9	21.0	0.0020	"Pitressin"	5.0	76.2
		38.0	0.0002	"Pitressin"	36.0	5.3
4	2.5	7.0	0.0002	"Pitressin"	3.0	57.1
		4.5	0 0004	"Pitressin"	2.5	44.4
5	2.2	33.0	0.0002	"Pitressin"	8.5	74.3
6	1.8	11.5	0.0002	"Pitressin"	0.2	98.2
		5.0	0.0001	"Pitressin"	1.5	70.0
7	1.8	14.8	0.0002	"Pitressin"	4.4	70.3
8[a]	1.9	26.6	0.0002	"Pitressin"	10.1	62.1
		24.4	0.0004	Unknown[b]	22.1	9.5
9[a]	2.1	22.1	0.0008	Unknown[b]	20.1	9.1
		22.1	0.0020	Unknown[b]	18.9	14.5
10[a]	2.0	15.6	0.0020	Unknown[b]	6.5	58.4
		25.2	0.0002	"Pitressin"	6.5	74.2
		25.2	0.0020	Unknown[b]	5.4	78.6
11[a]	1.9	11.1	0.0010	Unknown[b]	5.1	54.1
		47.9	0.0002	"Pitressin"	35.3	26.3

[a] In this table the last four rabbits were used (a total of 9 doses) to show that the unknown was relatively weak—certainly less than 50% of the standard on rabbit No. 8 and much more than 20% of the standard on rabbit No. 11. This result of 30% to 40% agrees quite well with the actual dilution of 40%

[b] The unknown tests less than 50% and more than 20% of the standard "Pitressin." Actually it was made 40% of the standard.

TABLE II.—COMPARATIVE ANTIDIURETIC AND PRESSOR ACTIVITIES OF DIFFERENT "PITRESSINS"

Sample No.	Rabbit Nos	Antidiuretic Activity	Pressor Activity
522X2A	15 and 21	0.4 Unit/cc.	0.35 Unit/cc.
522X2B	16, 22, 26, 30	2.0 Units/cc.	2.0 Units/cc.
522X2C	18	1.0 Unit/cc.	1.0 Unit/cc.
522X2D	19 and 23	> 0.3 Unit/cc.	0.3 Unit/cc
P 228	41, 43–47	0.005 Unit/cc.	< 0.05 Unit/cc.
P 229	36–40	1.5 Units/cc.	0.6 Unit/cc.
102459	51, 54, 59	14.0 Units/cc.	14.0 Units/cc.
102460	48, 49, 50	15 to 18 Units/cc.	11.0 Units/cc.
X2621	99, 100, 101	4.0 Units/cc.	6.0 Units/cc.
X2622	96, 97, 98	4.8 Units/cc.	4.8 Units/cc.
X2623	90 to 95	3 to 4 Units/cc.	4.8 Units/cc.
X2417	104, 106, 109 110–113	3.3 to 5.0 Units/cc.	5.0 Units/cc.
106294	102, 105, 107 109, 111, 112	4 to 5.5 Units/cc.	5.5 Units/cc.
86953	119–123	6.0 Units/cc.	6.0 Units/cc.
101686	119–123	5 to 6 Units/cc.	6.0 Units/cc.
522X14E	124–131	0.8 Unit/cc.	0.8 Unit/cc.

[1] "Pitressin" will be used throughout to designate the trade-marked name of the commercial product.

TABLE III.—INTERNATIONAL STANDARD, U. S. P. STANDARD AND "PITRESSIN" STANDARD

Rabbit No.	Wt., Kg.	Cc. Urine, Before	Dose, Unit	Cc. Urine, After	Antidiuretic Effect, %
55	2.6	11.0	0.0002 Pitr.	5.7	48.2
		38.5	0.0002 U. S. P.	15.6	59.5
56	2.0	13.0	0.0002 Pitr.	5.0	61.5
		21.8	0.0002 U. S. P.	14.0	35.8
57	1.9	30.3	0.0002 U. S. P.	14.3	52.8
		81.9	0.0002 Pitr.	24.2	70.5
61	2.0	44.1	0.0002 Int.	9.7	78.0
		79.7	0.0002 U. S. P.	6.8	92.5
63	2.0	18.5	0.0002 Int.	0.4	97.8
		22.3	0.0002 U. S. P.	11.8	47.1
64	2.2	34.6	0.0002 Int.	19.7	43.1
		83.0	0.0002 U. S. P.	35.7	57.0
65	1.8	32.1	0.0002 U. S. P.	2.8	91.3
		60.2	0.0002 Int.	14.1	76.6
66	1.8	9.4	0.0002 U. S. P.	2.6	72.4
		5.4	0.0002 Int.	1.2	77.8
69	2.7	28.7	0.0002 Int.	11.7	59.2
		76.6	0.0001 U. S. P.	62.6	18.3
70	1.6	27.6	0.0002 Int.	18.3	33.7
		39.8	0.0001 U. S. P.	37.3	6.3
71	1.7	34.0	0.0004 Int.	25.0	26.5
		32.5	0.0002 U. S. P.	28.5	12.3
72	2.2	21.8	0.0004 Int.	25.9	None
		25.6	0.0003 U. S. P.	45.7	None
73	1.9	199.9	0.0004 Int.	17.0	14.6
		31.5	0.0003 U. S. P.	24.9	21.0
74	2.0	15.0	0.0003 U. S. P.	10.4	30.7
		18.0	0.0004 Int.	28.3	None
76	2.8	19.6	0.0004 Int.	13.4	31.6
		45.9	0.0003 U. S. P.	29.8	35.1
77	1.8	39.7	0.0004 Int.	26.7	32.7
		29.7	0.0003 U. S. P.	21.2	28.6
		58.4	0.0003 Int.	57.0	2.4
78	2.0	11.7	0.0003 U. S. P.	4.6	60.7
		7.7	0.0004 Int.	5.9	23.4
		5.7	0.0003 U. S. P.	7.4	None
81	2.1	17.5	0.0003 U. S. P.	11.6	33.7
		15.7	0.0004 Int.	14.1	10.2
		12.4	0.0003 U. S. P.	10.9	12.1
82	1.8	19.8	0.0003 U. S. P.	23.1	None
		30.0	0.0004 Int.	34.5	None
84	1.8	6.2	0.0003 U. S. P.	5.0	19.4
		5.9	0.0004 Int.	6.6	None
88	2.0	17.5	0.0003 U. S. P.	11.5	34.3
		15.4	0.0004 Int.	14.8	3.9
		14.1	0.0004 U. S. P.	5.0	64.5

International Standards when the new lot was tried out before replacing the old (6). At that time the antidiuretic activity of the U. S. P. Reference Standard was not tested by us at least, but presumably when it was first set up by the U. S. P. Committee its various types of activity must have been checked against that of the International Reference Standard. Rabbits Nos. 69, 70, and 71 showed the International to be more than 50% as active as the U. S. P. Standard and Nos. 76, 77, and 81 showed its activity to be about 75%. It was therefore concluded that the antidiuretic activity of the present International Reference Standard is about 75% of that of the U. S. P. Reference Standard as determined by this rabbit method.

Table IV gives results on 6 rabbits, but only 4 were of any value in showing the effect of varying doses of the same product (the U. S. P. Standard Extract) on the same rabbit. While a dose of 0.00010 unit will give a definite effect especially if given first, the 0.0002 and 0.0003 unit doses are better adapted for use in quantitative assays as shown by rabbit No. 116. The 0.0004-unit dose is apparently too large although rabbit No. 117 shows a nice difference between doses of 0.0003 and 0.0004 unit. In general 4 doses given to the same rabbit are too many to give reliable results but 2 and sometimes 3 doses act quite well on the same animal.

Our results confirm the desirability of giving a large dose of glucose intravenously to start a diuresis (4) and also of giving 50-cc. intravenous saline infusions frequently but at regular intervals. After using water given directly into the stomach in fully 80% of our experiments we found that the omission of this procedure gave more accurate and more rapid comparative results. It is therefore recommended that this modification suggested by Fugo and Aragon (4) be omitted since it is not necessary if saline infusions are given to maintain diuresis.

SUMMARY AND CONCLUSIONS

1. The intravenous rabbit antidiuretic method for the evaluation of the antidiuretic activity of

A TYPICAL PROTOCOL IN DETAIL

Rabbit No. 121, wt. 1.7 Kg. March 3, 1948
3 mg./Kg. Morphine Sulfate subcu. at 8:30 a. m.
1.5 Gm./Kg. Urethane intraper. at 8:30 a. m.
25 cc. of 20% solution of Glucose intravenously at 9:30 a. m.
Doses are in total pressor units given intravenously.

	50 cc. Saline I.V. 10:00–10:15	0.0003 Unit 101686 50 cc. Saline 10:45–11:00	50 cc. Saline 11:30–45	0.0003 U. 86953 Saline 12:15–30
	Cc. Urine	Cc. Urine	Cc. Urine	Cc. Urine
	10:05 1.8	10:50 0	11:35 1.0	12:20 1.2
	10:10 2.7	55 0.5	40 0.5	25 0.5
	10:15 1.2	11:00 0	45 1.0	30 0.5
	10:20 0.8	11:05 0	50 0.8	
	10:25 1.0	10 1.0	55 1.5	
	10:30 0.8	15 0	12:00 0.8	
	10:35 0.7	20 0.8	05 2.0	
	10:40 0.6	25 0	10 1.8	
	10:45 0.6	30 1.0	15 1.2	1:00 2.0
Total	10.2 cc. 100%	3.3 cc. 32.35%	10.6 cc. 100%	4.2 cc. 39.6%

	50 cc. Saline 1:00–15	0.0003 Unit U. S. P. Std. Saline 1:45–2:00	50 cc. Saline 2:30–45	50 cc. Saline 3:15–30
	Cc. Urine	Cc. Urine	Cc. Urine	Cc. Urine
	1:05 1.3	1.50 0	2:35 1.0	3:20 0
	10 1.0	55 0.7	40 0.5	
	15 1.2	2:00 0.8	45 0	30 1.1
	20 1.0	05 1.0	50	
	25 1.2	10	55	
	30 0.8	15	3:00 0.9	45 1.2
	35 0	20	05	
	40 0	25	10	
	45 1.8	30 1.1	15 0.5	4:00 1.5
Total	8.3 cc. 100%	3.6 cc. 43.4%	2.9 cc.	3.8 cc.

Reduction in urine output to 32 35%, 39 6%, and 43.4% of the preceding normal indicates similar an diuretic activities for Nos. 86953 and 101686 in direct comparison with the U. S. P. standard extract.

TABLE IV.—EFFECT OF VARYING DOSES OF THE U. S. P. STANDARD ON THE SAME RABBIT

Rabbit No.	Wt., Kg.	Cc. Urine, Before	Dose, Unit	Cc. Urine, After	Anti-diuretic Effect, %
115	1.9	13.5	0.0002	14.6	None
		15.8	0.0001	15.9	None
		16.0	0.0002	30.9	None
116	2.0	22.7	0.0002	19.7	13.2
		38.6	0.0003	23.1	40.2
		35.1	0.0002	31.9	8.1
		14.2	0.0003	10.4	26.7
117	2.2	14.9	0.0004	6.4	57.1
		29.1	0.0003	45.1	None
		53.6	0.0004	37.0	31 0
		26.3	0.0003	14.9	43 3
118	2.0	26.5	0.0003	23.7	10.5
		47.7	0.0004	51.3	None
		61.1	0.0004	48.9	20 1
		19.3	0.0001	1.3	93.3
132	1.9	37.8	0.0002	28.2	25.4
		48.0	0.0003	34 3	28.5
		54.8	0.0004	49.4	10.0
		3.9	0.0001	1.8	53.8
133	1.5	7.0	0.0002	2.3	67.2
		3.0	0.0003	2.6	13.3
		4.1	0.0004	3.2	21.9
		10.6	0.0001	7.0	34.0
134	1.8	8.5	0.0002	8.2	3.5
		8.6	0.0003	20.4	None
		21.6	0.0004	35.8	None

posterior pituitary preparations has been found be more accurate and rapid than the previous used rat and mouse methods.

2. Certain modifications such as glucose an saline intravenously are valuable and are recon mended but the omission of the oral water dosag with its three-hour delay is desirable since it di not seem to contribute to the accuracy or un formity of our results.

3. The present International Reference Stanc ard while equal to the U. S. P. Reference Stanc ard in oxytocic and pressor activity was found t be only about 75 per cent as active by the rabbi antidiuretic method.

4. Two and often 3 doses may be directl compared in the same animal for antidiureti activity, but 4 doses, particularly if the fourth i larger than the others, will usually give an un reliable result.

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The Absorption and Penetration of Therapeutic Agents from a Carbowax Vehicle*

By DONALD B. MEYERS,† M. V. NADKARNI† and LOUIS C. ZOPF‡

The penetration of potassium iodide and phenolsulfonphthalein from "Carbowax" vehicles through the intact skin of albino rats has been studied. A change in rate and amount of penetration caused by the addition of silica, cetyl alcohol, wool fat, petrolatum, and "Tergitol" was demonstrated. Addition of cetyl alcohol and silica markedly reduced the absorption of the dye and the potassium iodide while the addition of petrolatum and wool fat caused a small decrease. Tergitol had little influence. A study also was made on the release of antiseptics from the same vehicles using a modified F. D. A. agar cup method. Absorption of phenolsulfonphthalein from the Carbowax base through the intact human skin was demonstrated clinically.

IT HAS BECOME well recognized that penetration and absorption of therapeutic substances from topical applications vary considerably with the type of vehicle employed. In recent years, many workers have met with success in preparing ointment bases which enhance the therapeutic activity of incorporated medicinal agents through increased absorption and penetration. The use of these newer type vehicles necessitates a study of the measurement and control of this increased activity.

Most therapeutic agents are not readily absorbed by the skin when they are incorporated in a base composed of fats, petrolatum, waxes, and oils. Previous investigators (1–4) have reported that the efficiency of antiseptics in oleaginous bases is much diminished or entirely absent. The addition of water and the presence of oxycholesterol greatly improve the antiseptic value of this type of base (5). This germicidal activity is further enhanced in some of the newer water in oil or oil in water emulsions such as Hydrophilic Ointment, U. S. P.

Anhydrous Carbowax ointment bases which have proved practical as vehicles for antiseptic agents have recently been developed in our laboratories and by other investigators (6, 7). Absorption and penetration of the therapeutic agent incorporated in this type of base is much greater than from the petrolatum and oily bases.

The advantages of increased absorption and penetration are obvious. Antiseptics are released more readily at the site of application; diffusion of the medicament into the diseased area is more rapid and complete; lower concentrations of active ingredients may be employed to obtain the same therapeutic results; and endodermal treatment through external application is possible. However, drugs incorporated in highly penetrating vehicles may tend to be more toxic or allergenic. Fatalities have been reported in the case of external application of sulfur (8) and of salicylic acid (9). For this reason, it is essential that means of evaluating and controlling absorption and penetration be available.

Many methods with numerous modifications have been suggested to determine the absorption of therapeutic agents from an ointment. Neuroth and Lee (1) have compiled a summary of the procedures used by various investigators. These procedures include both *in vitro* (10–13) and *in vivo* (14–16) methods of determinination. More recent is a method adopted by Clark (17) for testing diffusion of sulfa drugs from various vehicles. Though some correlation may be seen in the results of these workers, much of the literature is contradictory due to the variations in proce-

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dures and controls. Evaluations of penetration and absorption from ointment vehicles vary, not only with the method used, but also with the therapeutic agent employed in the experiment. Other factors such as temperature, relative solubility of the therapeutic agent, skin condition, etc., alter results.

The purpose of this study was to establish the comparative ratio of absorption of therapeutic agents from White Ointment, U. S. P., Hydrophilic Ointment, U. S. P. and a simple Carbowax vehicle. Also demonstrated was the alteration of absorption through the addition of other ingredients to the Carbowax base.

The Carbowax vehicle used in this study was a heat stable anhydrous base composed of equal parts Carbowax 4000W and polyethylene glycol 400W. Earlier work had shown it to possess a high index of antiseptic release when the F. D. A. agar cup method of antiseptic evaluation was employed. Our present study demonstrates the means by which, through simple addition of other ingredients, the absorption of therapeutic agents from this base can be altered.

To determine the absorption from these vehicles, a modified variation of the Macht (15) method was employed. In this procedure, the ointment is applied to the intact skin of the experimental animal and the active ingredient from the ointment is collected in the urine. A quantitative analysis for the ingredient is then made. In addition we used, as a basis for comparison, a modified agar cup method and a dye penetration method of our own. A preliminary clinical study was undertaken and an attempt was made to correlate the results.

EXPERIMENTAL

Vehicles employed in the following tests were White Ointment, U. S. P. XIII, Hydrophilic Ointment, U. S. P. XIII, and the simple Carbowax vehicle described earlier. The Carbowax base will be referred to as Base I. Absorption studies were also made from Base I with 2% Tergitol 4, Base I with 2% Silica,[1] Base I with 5% cetyl alcohol, Base I with 10% lanolin and Base I with 10% petrolatum.

Method 1.—This procedure was used to determine the rate of absorption. All test ointments contained 2% of phenolsulfonphthalein. Albino rats, free of any skin abrasions, were used as the test animal. A well-defined area, 3 x 3 in., extending on both sides of the spinal column between the forelegs and tail, was clipped using care not to damage the epidermis. Two grams of the test ointment was applied evenly over this area without unnecessary friction. The treated rats were wrapped with adhesive tape in such a manner that they could neither rub off or ingest any of the application. They were then placed in separate metabolism cages and their urine allowed to collect in graduated cylinders containing a solution of 5% sodium hydroxide. At the end of every hour, the urine was expressed from each rat and the time of the first red coloration of the alkaline solution recorded. The results may be seen in Table I.

TABLE I.—RATE OF ABSORPTION OF PHENOLSULFONPHTHALEIN THROUGH THE INTACT SKIN OF ALBINO RATS FROM VEHICLES LISTED

Vehicle	No. of Rats	Time in Hours of First Positive Test	Time in Hours of All Positive Tests	Mean Time of Positive Tests
White Ointment U. S. P.	6	...	None in 12	...
Hydrophilic Ointment U. S. P.	6	8	12	9.5
Base I	6	3	10	6.7
Base I with 2% Tergitol 4	6	5	10	6.7
Base I with 2% Silica	6	5	11	8.0
Base I with 5% cetyl alcohol	6	6	13	9.7
Base I with 10% lanolin	6	5	11	7.3
Base I with 10% petrolatum	6	5	10	7.3

It is not intended that the data of this test, alone, be considered conclusive, but it does reveal a variation in the average absorption rate from the various vehicles. Absorption from Base I is significantly more rapid than from either Hydrophilic Ointment, U. S. P. or from White Ointment, U. S. P., the latter giving negative results. Addition of Tergitol 4 to Base I, which might be expected to increase the absorption rate, did not show such results. Silica and cetyl alcohol added to Base I caused a marked retardation of absorption. These results also indicate that the addition of a small amount of petrolatum or wool fat (up to 10%) does not significantly retard the rate of absorption. The addition of petrolatum might prove advantageous for increasing heat stability without lowering the activity of the base.

Method 2.—In the second method, the procedure is similar to that of the first. The applications employed in this test were 10% potassium iodide ointments using the same vehicles as before. Five grams of the ointment was applied to the clipped area of rats which were then wrapped and placed in metabolism cages as in the previous experiment. The urine was collected at the end of six-, twelve-, and twenty-four hour periods, and analyzed for potassium iodide. The results in milligrams can be seen in Table II.

All results obtained from Method 2 can be correlated reasonably with those obtained from Method 1 with the exception of the ones obtained from Base I with 10% lanolin. In this series of experiments, the quantity of potassium iodide absorbed varied inversely with the time required for penetration of the dye as revealed by the first experiment. The Carbowax base with added wool fat gave unexplainable high absorption of potassium iodide. Previous workers have been unable to agree on how the addition of lanolin to vehicles effected absorption. Luff

[1] Microfine silica supplied by the Linde Air Products Company New York.

(10) and Sauerland (18) show decreased absorption from wool fat containing bases while Breslauer (11) and Brown and Scott (14) conclude that the addition of lanolin to vehicles causes an increased absorption.

TABLE II.—AVERAGE NUMBER OF MILLIGRAMS OF KI FOUND IN THE URINE OF RATS AFTER APPLICATION OF 5 GRAMS OF 10% POTASSIUM IODIDE OINTMENT WITH THE VEHICLES LISTED

Vehicle	No. of Rats	Mg. of KI Excreted in Hours 0–6	6–12	12–24	Total
White Ointment U. S. P.	6	0	0.7	9.1	9.8
Hydrophilic Ointment U. S. P.	6	8.2	10.3	15.6	34.1
Base I	6	19.7	20.2	44.7	84.6
Base I with 2% Tergitol 4	6	14.7	30.2	43.5	88.4
Base I with 2% Silica	6	4.6	9.7	23.1	37.1
Base I with 5% cetyl alcohol	6	6.0	7.1	19.3	32.4
Base I with 10% lanolin	6	21.3	23.3	70.6	115.2
Base I with 10% petrolatum	6	13.6	26.9	38.4	78.9

Absorption of potassium iodide from Base I was eight times as great as from White Ointment, U. S. P. and more than twice that shown by Hydrophilic Ointment, U. S. P. The results also show that absorption from the Carbowax vehicle (Base I) may be reduced by 50% through the addition of 2% silica or 5% cetyl alcohol. As in the first method, these results also show that the addition of 10% petrolatum does not retard absorption significantly from Base I.

Method 3.—The third method employed was an agar cup method. This procedure was used to see how well the results of an *in vitro* test of this type correlated with the *in vivo* tests of Methods 1 and 2.

Melted agar media were inoculated with a strain of *Staphylococcus aureus*, poured into agar plates and allowed to cool. A hole, 15 ml. in diameter, was cut out of the center of the agar with a sterile test tube. Two grams of the ointment containing a germicidal agent was placed into the cavity with a specially constructed syringe, so that it just touched the agar. The plates were then incubated at 35° for thirty-six hours and the zones of inhibition measured. The measurement recorded was the distance in millimeters from the ointment to the inside of the ring of bacterial growth.

Three different series of plates were made, Series A—5% resorcinol; Series B—Ceepryn 1:1000; and Series C—5% sulfathiazole. As Ceepryn loses its antiseptic activity in the presence of a strong anionic compound, results of Ceepryn inhibition were not obtained with Hydrophilic Ointment, U. S. P. which contains sodium laurel sulfate or with Base I containing added Tergitol 4. It has been brought to our attention that Quisno, Gibby, and Foter (19) have demonstrated that agar reduces the germicidal potency of quaternary ammonium salts. It is possible, therefore, that the limited zones of inhibition indicated with Ceepryn are in part due to the inhibition caused by agar.

Table III shows the results obtained from Method 3.

TABLE III.—AVERAGE WIDTH IN MILLIMETERS OF ZONES OF INHIBITED BACTERIAL GROWTH ON AGAR PLATES INOCULATED WITH *Staphylococcus aureus* AROUND THE OINTMENTS LISTED

Vehicle	No. of Plates	Antiseptic Agent: 5% Resorcinol, Series I	1:1000 Ceepryn, Series II	5% Sulfathiazole, Series III
White Ointment U. S. P.	3	0	0	5.0
Hydrophilic Ointment U. S. P.	3	6.0	...	10.3
Base I	3	6 3	5.0	16.0
Base I with 2% Tergitol 4	3	6.0	...	15.6
Base I with 2% Silica	3	4.3	3.0	4.3
Base I with 5% cetyl alcohol	3	8.6	2.0	9.6
Base I with 10% lanolin	3	5.6	4.6	7.0
Base I with 10% Petrolatum	3	7.0	2.6	7.0

A reasonable degree of correlation is evident between the results of the agar plate method and the results of the *in vivo* methods. High results were obtained from resorcinol in Hydrophilic Ointment, U. S. P. and in Base I with cetyl alcohol. This may be explained by the increased solubility of the resorcinol in the stearyl alcohol of the Hydrophilic Ointment and in the cetyl alcohol of the Carbowax base. Lower results were obtained from Base I with lanolin than might be expected from the results of Method 2.

Method 3 is easier to use and less time consuming than Methods 1 and 2 and does not introduce the factor of individual animal variation. However, assuming that Method 2 is the most reliable and most nearly approaches actual clinical results, there are some occasions when the third method gives erroneous results such as those experienced with the resorcinol. There is not sufficient variation in penetration time to make Method 1 a reliable criterion of absorption.

In an initial clinical study, we were able to show absorption of phenolsulfonphthalein through the human skin from the Carbowax vehicle. Five grams of Base I containing 8% of the dye was applied to the patient's side and covered with a plastic bowl. Marked coloration from the dye was obtained in the alkalinized urine of all twelve patients tested, the concentration varying with the condition of the skin. Further clinical evidence is being compiled to substantiate the results of the other experimental data.

By combining the results of all the tests employed, the following conclusions may be drawn.

(1) Absorption of the therapeutic agents employed in this study is better from the Carbowax vehicle than from Hydrophilic Ointment, U. S. P., absorption from Hydrophilic Ointment being much greater than from White Ointment, U. S. P.

(2) Absorption of phenolsulfonphthalein and potassium iodide from a Carbowax base of this type

can be markedly reduced by the addition of 2% silica or 5% cetyl alcohol.

(3) The addition of a wetting agent such as Tergitol 4 is of little value for increasing absorption of phenolsulfonphthalein or potassium iodide from an anhydrous Carbowax base.

(4) Up to 10% of petrolatum may be added to the Carbowax vehicle without noticeably affecting the penetration rate of phenolsulfonphthalein or potassium iodide.

(5) The absorption results obtained by the addition of lanolin to the Carbowax base vary with the method.

SUMMARY

(1) The penetration and absorption of therapeutic agents from White Ointment, U. S. P., Hydrophilic Ointment, U. S. P., and from a Carbowax Base have been studied comparatively using three methods.

(2) A change in absorption value from the Carbowax base after addition of Tergitol 4, silica, cetyl alcohol, petrolatum, and wool fat has been demonstrated.

(3) Methods of retarding the absorption of phenolsulfonphthalein or potassium iodide from the Carbowax base by simple addition of cetyl alcohol or silica have been pointed out.

(4) Further clinical studies are in progress to learn which of the three methods outlined most nearly coincide with clinical findings.

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Book Reviews

Kommentarer Till Svenska Farmakopén, 1946. By WOLMER BONDESON, *et al.* Published by the Apotekarsocietetens Direktion, Stockholm, Sweden, 1947. 552 pp. 14.5 × 21 cm.

Evidence on all sides points to the fact that pharmacopœias all over the world have the same trend toward the inclusion of more potent and specific medications, the only difference being one of degree. Another important trend is the constant improvement of manufacturing methods leading to smaller amounts of impurities, which of course demands more sensitive means of detecting and determining them.

All of these trends lead up to a need, on the part of pharmacists and those scientifically trained persons who use pharmacopœias only occasionally, for adequate commentaries which explain the bases for these tests and standards. All too often when such commentaries are consulted, the reader finds a great deal of data which are useful concerning pharmacology, posology, and toxicology, but frequently only a limited amount of information on the chemistry involved in the standards.

This commentary on the Swedish Pharmacopœia of 1946 is devoted almost entirely to a discussion of the chemistry involved in this Pharmacopœia. Although the difference between Swedish and American standards is frequently rather great, this book will prove to be quite useful to anyone for whom the language is not a handicap.

Reference Book of Pharmaceutical Registration and Label Requirements in Latin America. Compiled by ALBERT AVIGDOR. James A. Marshall, Ltd., 47 West 56th Street, New York, 1948.

This handy little book gives, in outline form, the registration and label requirements for pharmaceuticals in each of the Latin American countries. The arrangement is alphabetical by country.

Under each country, the first page is a data sheet where the user of the book may record such useful information as consular fees for certification of documents, exchange rate, and names and addresses of firms handling registration matters.

The second page deals with Board of Health requirements and tells at a glance which products must be registered, how samples must be submitted how labels must be submitted, how packages are to be registered, all data and documents to be submitted, fees, validity, and prerequisites.

The third page, under each country, is concerned with label requirements. On this page will be found a capitulation of all statements required on labels.

The same plan of presentation is used for each country. The book has a loose-leaf form so that it can be modernized by means of a subscription system.

Since the author of this book was for many years in charge of the registration and label department of a large international drug firm, the information can

be assumed to be accurate. Certainly it is useful information for those exporting drugs to our southern neighbors.

Technic of Medication. By AUSTIN SMITH. J. B. Lippincott Company, Philadelphia, 1948. xi + 255 pp. 11.5 x 19.5 cm. Price $4.00.

Most pharmacists remember the late Bernard Fantus' book, *General Technic of Medication*, the third edition of which was published in 1938, and have wished for a modern treatise to take its place. Dr. Austin Smith has provided that book in an admirable manner.

Technic of Medication is an exceedingly practical book for physicians and others handling drugs and certain therapeutic appliances. Frequently Dr. Smith followed the same pattern as Dr. Fantus, but always the modern viewpoint is projected. An exemplary balance between therapeutic nihilism and the conservative, scientific use of drugs is maintained throughout. As expected, the physician is constantly reminded to use U. S. P., N. F., and N. N. R. drugs in the interest of sound and economic therapy.

Dr. Smith sympathetically discusses the role of hospital pharmacy. At other points will be found a frank discussion of two very old professional problems—the prescribing pharmacist and the dispensing physician. Dr. Smith rightfully concludes that both of these groups have only a limited place in medical care today. The ownership of prescriptions, the telephoned prescription, prescriptions by mail and other problems of inter-professional relations are discussed in an open and fair manner.

Following chapters on general principles, Dr. Smith takes up oral administration of drugs, parenteral administration, rectal and genito-urinary administration, applications to the skin, and mucous membrane applications. Each of these phases is covered briefly but accurately and practically. A final chapter deals with such practical aids as storage of drugs, physical aids, care of equipment, and removal of stains.

It is unfortunate that Dr. Smith curtailed the section on writing the prescription. If this section were more complete, it would make the book an ideal text in prescription writing for medical students. Helpful as the book is, supplementation is necessary in the classroom in order to teach fundamentals of prescription writing to the completely uninitiated.

Ownership of a copy of *Technic of Medication* should enable one to discuss fluently dosage programs, drug absorption, applications to the skin, the rational choice of a drug, and hundreds of other practical matters. A knowledge of the book's contents should also be helpful in advising patrons about the storage of medicinals, the care of insulin syringes, the administration of drugs to children, and other problems.

The British Pharmacopœia, 1948. Published under the General Council of Medical Education and Registration of the United Kingdom, London. Constable and Co., 1948. xl + 914 pp. 14.5 x 23 cm.

The publication of a pharmacopœia is always an important event to pharmacists and the publication of such an important pharmacopœia as the British is doubly eventful.

Since the last British Pharmacopœia was published in 1932, the character of pharmaceutical progress over the past fourteen years is reflected in the types of admissions. Most of the fresh infusions, 17 tinctures and 4 plasters were relegated to antiquity, while 57 injections, 18 tablets, 10 vaccines, and 9 sex hormones entered the stage.

In six instances the strength of official substances was changed. Of especial significance is the change of hydrogen peroxide solution to 5 to 7 per cent which is about double that of the U. S. P.

The general style and format has been changed to coincide more nearly with that of the U. S. P. Latin titles still take precedence, however.

No very significant changes were made in the inorganic drugs.

As would be expected, the changes in the organic field are too numerous to mention. The newly admitted alkaloids were colchicine, ergometrine maleate, ergotoxine ethanesulfonate, and papaverine hydrochloride.

In the field of local anesthetics, butacaine sulfate, butyl aminobenzoate, cinchocaine hydrochloride, benzyl alcohol, and bromethol (tribromethyl alcohol in amylene hydrate) have been added.

In the arsenicals, the dose of neoarsphenamine has been reduced and carbarsone has been newly admitted.

No new barbiturates were added; one acridine antiseptic, aminacrine hydrochloride; one new sulfonamide, succinylsulfathiazole; and the 10 sulfonamides previously recognized by supplement were added.

In the galenical field many changes were made. Tannic acid ointment, chrysarobin ointment, and hydrous ointment of ammoniated mercury were deleted. New ointment bases, an emulsifying ointment, unguentum emulsificans (emulsifying wax 30, white petrolatum 50, and liquid paraffin 20), and a hydrous emulsifying ointment, unguentum emulsificans aquosum (add 1 Gm. of chlorocresol in 699 Gm. of water to 300 Gm. of the former ointment), were admitted. Cetostearyl alcohol and sodium lauryl sulfate were also admitted.

No new solutions were added and 9 were deleted.

Four new suppositories have been added—bismuth subgallate, cocaine hydrochloride, extract of hamamelis, and the latter plus zinc oxide.

No new syrups were added, but Eaton's Syrup and syrup of ferrous iodide were deleted.

The changes in the vegetable drug field are all relatively minor. Surely no one will miss the few deletions such as tamarind.

It is interesting to note that penicillin is defined as "either the sodium salt or the calcium salt of the antimicrobial acid, which is produced when *penicillium notatum* or related organisms are grown under appropriate conditions or in a suitable culture medium." The bioassay does not seem to be as completely described as that of the Food and Drug Administration of this country. Official penicillin preparations are the injection, oily injection (peanut oil-beeswax), cream, sterilized cream, ointment, ophthalmic ointment, and lozenges.

Under injections, the method of handling "dry-filled ampuls" is unique and may be illustrated by Injectio Hexobarbitoni Sodii. This injection is

defined as "a sterile solution of Hexobarbitone Sodium in Water for Injection which is free from carbon dioxide. It is prepared by dissolving the contents of a sealed container in the required amount of water for Injection immediately before use."

The number of tablets increased from 1 in 1932 to 49 in 1948. In addition to tablets of the Seventh Addendum, 18 new ones were added. Perhaps the most unique feature about the tablet monographs is that in addition to listing "usual sizes," a specific dosage is to be dispensed if the physician does not specify. For example, under Tabellae Acidi Ascorbici it advises, "If the quantity to be contained in a tablet is not stated, tablets containing, in each, 50 mg. shall be dispensed or supplied." In addition weight limitations are placed upon individual tablets based on a percentage of the average of the sample.

All in all, the British have done a commendable job in their Pharmacopœia of 1948 under conditions far more difficult than we have ever experienced. Congratulations from all pharmacists, the world over, are in order.

Aquametry. *By* JOHN MITCHELL JR., and DONALD MILTON SMITH. Interscience Publishers, Inc., New York, 1948. xi + 444 pp. 15 x 23 cm. Price $8.

During recent months, analytical chemistry has been struggling in the face of the dramatic discoveries of organic and physical chemists to make the scientific public more conscious of its importance. The series on *Chemical Analysis* published by the Interscience Publishers has been of material assistance in that regard. Edited by B. L. Clarke and I. M. Kolthoff and with such men as Ashley, Sandell, Willard, and others as an advisory board, the series on monographs of modern chemical analysis has been welcomed by many. *Aquametry* is Volume V of the series.

The finding of moisture in all sorts of substances has been a very important determination. In many analytical laboratories a drying procedure is probably carried out more frequently than any other test. Oven drying, distillation, and many other techniques have been used for this determination. But it was not until the introduction of the Karl Fischer method in 1935 that a precise chemical method was available.

That the Karl Fischer method is of value is evidenced by the voluminous literature recorded on the subject during the fourteen years since its introduction. However, the application of this method is frequently not easy. To evaluate the method critically in book form provides, therefore, a monograph, exceedingly valuable to analytical and control chemists.

This book on Aquametry, a name coined by the authors, is divided into two parts. Part 1 deals with the specific quantitative determination of water by the Karl Fischer reagent. The nature of the reagent is discussed as well as the general directions for its use in different methods. General applications of the reagent to determine not only moisture, but functional groups, are discussed. The last chapter in the section is given over to the determination of water in commercial organic materials.

Part 2 deals with organic reactions involving the liberation or consumption of water. Here one finds separate chapters on the determination of alcohols, carboxylic acids, amines, peroxides, etc.

A unique and helpful feature of this useful book is a chapter on proposed further studies. Here one finds many desirable projects in both the fundamental and applied fields.

The book is well documented and both subject and author indexes appear to be carefully done. The paper, binding, and general workmanship of this book are of the usual high quality found in this series.

The Literature on Streptomycin, 1944–1948. By SELMAN A. WAKSMAN Rutgers University Press, New Brunswick, N. J. 1948. xv + 112 pp. 15 x 23 cm. Price $3.

Although the isolation of streptomycin was announced in January, 1944, literature has accumulated about 1200 scientific papers during the intervening years. It is fitting, therefore, that the senior discoverer of streptomycin should assemble the references for those interested.

This book opens with a reproduction of the first paper announcing streptomycin as it appeared in the *Proceedings of the Society for Experimental Biology and Medicine*, 55, 66–69, 1944. This is followed by a selective list of general references on the actinomyces, their antagonistic properties, and on streptothricin. Following this, appear 1171 references to streptomycin arranged in chronological order. An author index and a subject index complete the book.

Die Chemische Industrie und ihre Nebenprodukte. Verlag für wirtschaftsaliteratur G.m.b.H., Burstwiesenstrasse 53, Zürich, 3. 1947. 253 pp. 14.5 x 22.5 cm.

To those who have occasion to use information concerning Swiss chemical firms, this directory will be useful. The directory contains an extensive list of the Swiss chemical and allied industries, arranged according to products. Names and addresses of each firm, leading executives, dates of establishment, banking connections, products and brands, and other essential information are given.

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Muscle Irritation Following the Injection of Various Penicillin Preparations in Rabbits*

By A. A. NELSON, C. W. PRICE and HENRY WELCH

SINCE the introduction of penicillin as a chemotherapeutic agent numerous preparations have been devised which were designed to delay its absorption from the tissues and thus prolong the effective concentration of penicillin in the blood. Penicillin in oil and wax was one of the first preparations (1) which successfully accomplished this purpose. This product consists of either calcium penicillin or crystalline sodium or potassium penicillin incorporated in a menstruum of peanut or sesame oil and wax. This admixture of penicillin with oil and wax resulted in a delayed absorption of the highly soluble penicillin salt and prolonged penicillin concentrations for twenty-four hours in most individuals injected intramuscularly with 300,000 units. Recently, prolongation of penicillin blood concentrations has been accomplished by using procaine penicillin (2, 3). Procaine penicillin is a relatively insoluble salt of penicillin, and, at present, is used in three dosage forms: procaine penicillin for aqueous injection, procaine penicillin in oil, and procaine penicillin in oil with aluminum monostearate (4). It is possible with the latter product to obtain blood concentration of penicillin for about four days following the injection of 300,000 units. The usual method for administration of these preparations is by the intramuscular route.

During the time that penicillin in oil and wax was widely used numerous complaints were voiced concerning the pain following intramuscular injection, the occasional formation of sterile abscesses, and the persistence of lumps due to the slow absorption of the beeswax. The intramuscular injection of procaine penicillin preparations is relatively painless, due probably to the analgesic effect of the procaine. This lack of pain would not, however, lessen any possible muscle damage caused by the procaine penicillin or other constituents of the product. Accordingly, it was thought desirable to compare the pathological effects of various penicillin products on rabbit muscle. This study includes the preparations already mentioned, the vehicles used in compounding them, and, in addition, other penicillin products less widely used for the prolongation of penicillin blood concentrations. The

* Received Dec. 31, 1948, from Federal Security Agency, Food and Drug Administration, Washington, D. C.

formulas of the products used in this study are given in the following table:

1. Procaine penicillin in sesame oil with 2 per cent *w/v* aluminum monostearate.
2. Procaine penicillin in sesame oil.
3. Procaine penicillin, aqueous suspension, with about 0.5 per cent carboxymethyl cellulose.
4. Vehicles used with procaine penicillin, 2 per cent sodium alginate, 2 per cent *w/v* aluminum monostearate in sesame oil.
5. Penicillin in oil and wax.
6. Aluminum penicillin in oil.
7. Penicillin in oil with epinephrine.
8. Procaine penicillin in oil with pectin and 2 per cent *w/v* aluminum monostearate.

One milliliter of the preparation under test was injected into the sacrospinalis muscle of the rabbit. All of the penicillin products contained 300,000 u./ml. By using the anterior and posterior portions of each muscle it was possible to test four samples per animal. The animals were sacrificed for the most part at seven or at fourteen days, the muscles removed and fixed in 10 per cent formalin.

PATHOLOGY

There were submitted for pathological examination 58 blocks of rabbit muscle fixed in formalin, each block containing an injection site. After gross examination of each muscle block, paraffin sections were made and stained with hematoxylin-eosin. The pathologist did not know the identity of the materials injected.

On both gross and microscopic examination distinct differences were observed between the effects produced by certain categories of the injected compounds, while the lesions caused by others were quite similar in appearance. Unless otherwise stated, the appearances associated with any one compound were not greatly dissimilar in the seven-day as compared with the fourteen-day specimens, and one description suffices for both.

Procaine penicillin in sesame oil with 2 per cent *w/v* aluminum monostearate caused areas of muscle damage averaging about 2.5 x 0.5 x 0.5 cm. The color of the damaged areas, after formalin fixation, was light yellowish brown. Small to moderate numbers of cystic spaces 1 mm. or less in diameter contained a fluid or semifluid colorless oily material. Half the specimens in this group also contained small amounts of creamy whitish material. In a third of the specimens much of the area of injection was of a friable and semifluid character. Microscopically, the injection sites showed moderate amounts of muscle necrosis, small cystic areas containing more or less of amorphous or necrotic debris, inflammatory cellular exudate, fibrosis, and foreign body giant cells. Hemorrhage and calcification were present in only small amounts.

Procaine penicillin in sesame oil caused changes very similar to those described in the previous paragraph, but the degree of damage was possibly just a little less.

Aluminum penicillin in oil, penicillin in oil with epinephrine, and procaine penicillin in oil with pectin and 2 per cent *w/v* aluminum monostearate, all caused changes about as described above; however, material submitted for examination was limited in amount in comparison with the other categories of injected material discussed in this paper.

Penicillin in oil and wax caused lesions whose size was the same as with the compounds described above, but which differed moderately on both gross and microscopic examination. The consistency was firmer, and there was a slight grittiness to touch. Very few small cystic spaces and no creamy material were noted. Microscopically, there was more fibrosis and more walling-off of necrotic muscle than resulted with the compounds described above, and somewhat more numerous foreign body giant cells, while there was less of acute inflammatory cellular exudate.

Very little muscle damage was noted in the fourteen-day specimens following administration of procaine penicillin, aqueous suspension with about 0.5 per cent carboxymethyl cellulose. The lesions at seven days were intermediate in size between the fourteen-day ones and those described in previous paragraphs. No creamy material was noted grossly. Qualitatively, the microscopic appearance did not differ much from that in the penicillin in oil categories, but quantitatively it correlated with its gross appearance in being definitely decreased in amount.

The two vehicles injected separately, namely 2 per cent sodium alginate and 2 per cent aluminum monostearate in sesame oil, showed grossly only small amounts of reddish streaking in the muscles. Microscopically, there was no calcification or foreign body giant cells; other changes as previously noted were present in very small amount. A basophilic jelly-like material was present between bundles of muscle fibers in the alginate specimens. The total amount of damage from the vehicles alone was very small.

COMMENTS

On pathological examination, none of the penicillin preparations included in this study caused more than moderate damage to rabbit sacrospinalis muscle when injected in 1-cc. doses.

All the penicillin in oil preparations, with or without procaine and/or aluminum monostearate, caused gross and microscopic appearances of a quite similar nature.

Penicillin in oil and wax showed damage similar in extent to that caused by the substances just mentioned, but with certain gross and microscopic differences.

Aqueous procaine penicillin caused damage intermediate in amount between that in the above categories and that from the vehicles alone. Damage from the latter was very slight.

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A Study of the Reported Condensation of Chloral with *p*-Chlorobenzenesulfonic Acid. An Improved Synthesis of *p*-Chlorobenzenesulfonic Acid*

By WALTER A. COOK and KATHRYN H. COOK†

The synthesis of *p*-chlorobenzenesulfonic acid has been studied and a preparative method for this compound is described in detail. Three separate methods for the condensation of chloral with *p*-chlorobenzenesulfonic acid are described and evaluated.

A STUDY of the literature on the synthesis of DDT indicated that while many condensing agents have been considered, only sulfuric acid (11), chlorosulfonic acid (4, 13), and hydrofluoric acid (15) have been successfully employed in its preparation. The technical production of DDT has been limited to the exclusive use of the first-mentioned condensing agent, even though it was pointed out recently that the economic operating limit of this method as expressed in terms of the percentage theoretical yield of pure DDT, is appreciably lower than that by the chlorosulfonic acid method (5). In 1945 an article by Giral and Rangel (7), in an obscure periodical, announced the synthesis of DDT in quantitative yield from chloral and *p*-chlorobenzenesulfonic acid according to the following reaction: $CCl_3CH(O)+2p\text{-}ClC_6H_4SO_3H \rightarrow H_2S_2O_7 + CCl_3CH(p\text{-}C_6H_4Cl)_2$.

Moreover, these authors postulated that the condensation of chloral with chlorobenzene in the presence of sulfuric acid occurs through the formation of an intermediate compound, *p*-chlorobenzenesulfonic acid and therefore they believed the quality of the DDT technical product might be improved through the direct synthesis of the insecticide according to the above-indicated equation.

The singular nature of this claim together with the long-recognized stability of the C—S bond in aryl sulfonic acids, suggested to the authors that this work be re-examined to determine whether it could be substantiated.

EXPERIMENTAL

Since the literature references on *p*-chlorobenzenesulfonic acid (1, 2, 6, 10, 12) disclosed no information as to yields, and no actual isolation or purification of the free acid from either the sulfonation reaction product or from any of the several salts prepared and described, the development of a satisfactory synthetic procedure for *p*-chlorobenzenesulfonic acid became necessary as a preliminary to the intended condensation studies of this compound with chloral. Accordingly, the details of the preparation of *p*-chlorobenzenesulfonic acid are given as follows:

In a three-necked flask fitted with a mercury seal stirrer, dropping funnel, thermometer, and gas exit tube, was placed 225 Gm. chlorobenzene (2 moles). The flask and contents were cooled to 0° in an ice-salt water bath and 230 Gm. chloro-

* Received Oct. 26, 1948, from the Knight Chemical Laboratory, University of Akron, Akron, Ohio.
† The authors acknowledge the courtesy of Mr. Hector H. McAllister for translation service, and Mr. F. von Bergen of the Westvaco Chlorine Products Corporation for the sample of technical chloral, used in this investigation.

sulfonic acid (2 moles) added dropwise over a sixty-minute interval with the temperature of the reaction mixture being maintained at 10–15° for a total stirring period of six hours. The contents of the flask were then poured into 500 ml. saturated NaCl which in turn was surrounded by an ice salt bath. The precipitated sodium *p*-chlorobenzene sulfonate was filtered on a Büchner funnel, and air-dried to a constant weight of 377 Gm. (theoretical yield, assuming the anhydrous salt form, is 429.2 Gm.). The monosodium salt was then transferred to a 1-liter beaker, 500 ml. 95% ethyl alcohol and 294 ml. concentrated HCl (3.5 moles) were added and the mixture warmed at 50–60° for two hours before removal of the sodium chloride by filtration. The filtrate was evaporated to dryness with a subsequent yield of 200 Gm. crude *p*-chlorobenzenesulfonic acid. This product after dissolving in 100 ml. glacial acetic acid, filtering, cooling, and adding 175 ml. $CHCl_3$ gave 160 Gm. or a 41.5% theoretical yield of pure acid (dried in a desiccator over KOH pellets, and concentrated H_2SO_4) which subsequently melted at 93° (corr.) and checked with *the revised m. p. of Tanasescu and Macarovici* (17). Moreover, a test portion of the acid was converted into the sulfonchloride and the latter into the amide and the observed melting points of both derivatives were found in agreement with the accepted literature values. Strangely enough, even though the revised melting point for the acid (17) was first announced in 1938, the incorrect value of 68° (first reported by Krafft and Wilke (10) in 1900), which is the accepted m.p. value for the hydrate, still persists in such reference sources as Heilbron (8), Kamm (9), Shriner and Fuson (14), and Cheronis and Entrikin (3) although all such sources were printed since 1938. Suter (16) neither cites the work of Tanasescu and Macarovici as a reference, nor does he give either m. p. value for *p*-chlorobenzenesulfonic acid or its monohydrate.

CONDENSATION STUDIES

Three separate experimental attempts were undertaken to condense chloral with *p*-chlorobenzenesulfonic acid. The first and second runs were made under conditions simulating those meagerly described by Giral and Rangel (7). Thus, 0.2 mole of *p*-chlorobenzenesulfonic acid and 0.1 mole of technical chloral were mixed without any perceptible heat effect. After heating the mixture under reflux for three hours on a water bath it was poured into 300 Gm. crushed ice. The reaction mixture was completely miscible and thus the solution was evaporated to dryness. A strong odor of chloral was observed during the evaporation and the grayish residue was dissolved in 100 ml. water and poured into 300 ml. chilled saturated NaCl solution with the resultant precipitation of *p*-chlorobenzenesodium sulfonate which after filtering and drying, weighed 38.5 Gm. (0.18 mole), a recovery of 90% of the original *p*-chlorobenzenesulfonic acid used in the experiment.

A parallel run in which the same quantities of reactants were employed (except in this case the chloral was freshly prepared by dehydration of chloral hydrate with concentrated H_2SO_4 overnight) yielded under similar conditions, a water-insoluble fraction when poured onto ice. After filtration, the filter cake was suspended in a solutio of 25 ml. of ethanol and 100 ml. water, and aft refiltering and drying in air, weighed 8 Gm. Th material on a block melting point determinatio indicated it was not DDT by softening at 13 and melting with slow decomposition at 145–17 with the simultaneous liberation of a pronounce odor of chloral. From its behavior it was believe to be a water-insoluble chloral-polymer. The add tion of 300 ml. chilled saturated NaCl solutio to the filtrate from the original water-insolubl crude filter cake yielded 41.5 Gm. dry sodium *p* chlorobenzenesulfonate (theoretical weight equiva lent to 0.2 mol. *p*-chlorobenzenesulfonic acid = 42.9 Gm.).

In a third run *p*-chlorobenzenesulfonic acid wa first prepared as previously described for th synthesis of the acid, and then without its separatio from the reaction mixture, treated subsequentl with chloral in order to further establish whethe DDT could be synthesized as claimed by Giral an Rangel. *Thus 1 mole chlorobenzene and 1 mol* of chlorosulfonic acid were reacted and then 0. *mole of technical chloral added dropwise over* twenty-minute interval at a temperature of 10° Stirring was continued for four hours and the re action product allowed to stand overnight at roon temperature. It was then poured into crushed ic and after filtration and drying, 91 Gm. of crud water-insoluble filter cake was obtained. Th latter was washed alternately by decantation wit 150 ml. cold ethyl alcohol and 150 ml. cold petroleu ether and after the second filtration, 49 Gm. of in soluble fraction remained. The latter, upon tw successive recrystallizations from a mixture o $CHCl_3$ and petroleum ether was identified by it melting point of 145° as *bis*-(*p*-chlorophenyl) sulfone The original acid filtrate from the water-insolubl reaction product upon treatment with chilled sodiu chloride solution as previously described yielded 120 Gm. air-dried *p*-chlorobenzene sodium sulfonate. On the basis of the 49 Gm. crude sulfone (0.18 mole) or an equivalent 0.366 mole original chlorobenzene and 120 Gm. sodium *p*-chlorobenzenesulfonate (0.558 mole) a total of approximately 93% of the original chlorobenzene was accounted for. Thus, from the three runs described above there is no evidence for the formation of DDT from *p*-chlorobenzenesulfonic acid as claimed by Gira and Rangel (7). Since it has been demonstrated that DDT can be prepared by the addition of chlorosulfonic acid to a mixture of chlorobenzene and either chloral or chloral hydrate (4), and since it has also been shown that mono and dialkylated derivatives of aromatic hydrocarbons can be synthesized in a similar manner from the corresponding alcohols and appropriate aromatic hydrocarbon (13), it is obvious that the postulated mechanism of Rueggeberg (13) rather than that of Giral and Rangel (7) is in accord with all the experimental evidence presented for the synthesis of DDT.

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Mentha Cardiaca. I. Pharmacognostical Studies and a Preliminary Investigation of the Volatile Oil*

By KEITH D. BALLENTINE and ARTHUR E. SCHWARTING

A report of the histological, morphological, and growth characteristics of *Mentha cardiaca.* Chemical and physical constants of the oil are presented and comparisons with spearmint and its oil are made.

THE PLANT, *Mentha cardiaca* Gerarde, commonly called Scotch Mint and Little Leaf Mint, is a perennial *Labiatae* resembling *Mentha spicata* in structure and odor. The fact that it was observed to produce a volatile oil in a high percentage and that the volatile oil resembles spearmint oil prompted the present study. The available literature disclosed no chemical or histological investigation of the plant.

The plant is tall and erect with pubescent stems and leaves. The leaves are petiolate, unequally serrate, oblong-to-ovate-lanceolate with an acuminate apex. The leaf color is somewhat lighter green than that of spearmint, and the purple color which often appears on the basal stem of spearmint is not observed on *M. cardiaca.* The flowers exist in interrupted whorls in the axils of the leaves, the corolla being pale purple in color. The flowering dates parallel those of *M. spicata.*

EXPERIMENTAL

Cultivation.—Root cuttings were introduced to the medicinal plant garden in 1944. The area was expanded in 1945 and distillations were made in that year and in the following year from an area 50 feet square. In the spring of 1947 a systematic investigation of this and three other mints was begun and two areas of cultivation were established; the one a typical dry land area, and the other a fertile lowland with irrigation facilities. The main observations and data presented in this paper were obtained from these latter plantings. Four randomized plots of five 18-foot rows each of *Mentha cardiaca* appeared in each area. The rows were planted two feet apart, the single cuttings being planted one foot apart in the row.

The plant demonstrated unusual vigor, the root system expanding more rapidly than spearmint or peppermint. The crop was taken in the last week in July and at that time it was already difficult to outline single rows. A 16-foot length of the center row of each plot was collected and the weight recorded. These data were used in the preparation of Table I. The remaining plot area was also harvested and the total material of the four plots of each area was separately distilled. Because no significant difference regarding plant or oil yield from the plots of the two areas was observed the data appear as an average of all materials.

Distillation.—Three hundred forty pounds of the overground flowering plant was distilled in lots of about 50 pounds each. The plants collected in mid-morning were allowed to wilt in the field and distillation was initiated immediately and continued until all material was distilled. The aqueous waters were subjected to cohobation processes in a separate still and the oil collected. The oils were not mixed. Each was dried, filtered, and stored in tightly stoppered and filled containers. The yield, expressed as per cent, in Table I is the total oil.

TABLE I.—YIELD DATA OF *Mentha cardiaca*

Fresh Plant per Acre, Lbs. (Calculated)	Oil Content, %	Oil Yield per Acre, Lbs. (Calculated)
12,378	0.477	59.0

Leaf Histology.—Leaf and stem material was collected and fixed in a formalin-acetic acid-alcohol solution. A specimen of *M. spicata* was similarly prepared for a comparison of structures. The materials were embedded in paraffin and sectioned with a rotary microtome. Finished permanent slides were prepared of all specimens. Hand sections of these and fresh materials were also prepared.

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Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.

The lamina (Figs. 1 and 2) is thin and consists of the epidermal layers, the chlorenchyma, consisting of a single row of irregular palisade cells and a spongy region of three or four layers of cells. Both glandular and nonglandular trichomes occur on the leaf surfaces, being more numerous on the dorsal surface and on the veins. The uniseriate multicellular trichomes vary in length from 85 to 180 microns. Numerous single-celled trichomes occur. Glandular trichomes of the stalked type are numerous. The stalk is one or two celled with a globular gland composed of one cell up to 24 microns in diameter. Sessile glands are less common and are found in epidermal depressions. The gland is six to eight celled.

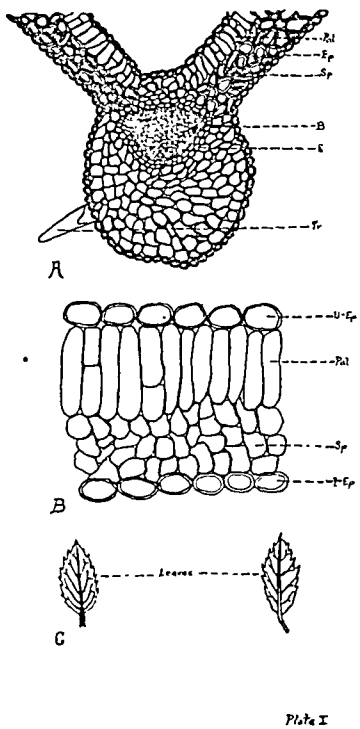

Fig. 1.—*Mentha cardiaca* Gerarde. T. S. of leaf. *A*, midvein; *B*, lamina. *U-Ep*, upper epidermis; *L-Ep*, lower epidermis; *Pal*, palisade cells; *Sp*, spongy parenchyma; *B*, fibrovascular bundle; *S*, sieve; C, leaves.

A peculiar epidermal structure is illustrated in Fig. 2 (*D*). It appears as an epidermal eruption and is surmounted by two uniseriate trichomes. The structure is not common.

Stomata are more numerous in the dorsal epidermis of the lamina and are somewhat larger in the ventral surface. Two or three contiguous cells surround the guard cells and occasionally the stomata appear capped as illustrated in Fig. 2 (*E*).

Stem Histology.—The stem (Fig. 3) is an amphiphloic siphonstele structure, typical of the *Labiatae*. A cuticle covers the epidermal cells and numerous uniseriate trichomes and a few glandular trichomes

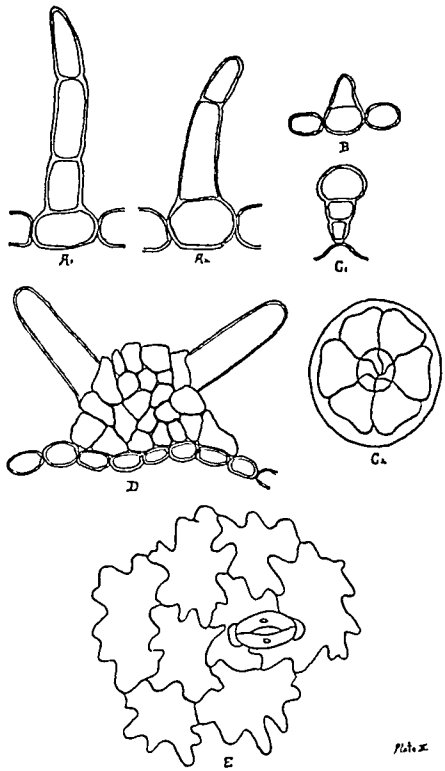

Fig. 2.—*Mentha cardiaca* Gerarde. Epidermal structures. A_1, A_2, *B*, uniseriate nonglandular trichomes; C_1, C_2, glandular trichomes; *D*, epidermal structure; *E*, lower epidermis of lamina showing stomata.

occur. The former measure up to 200 microns in length and are composed of as many as 7 cells.

A comparison of these structures in a typical specimen of *M. spicata* demonstrates several differences. Cardiaca trichomes of the nonglandular type are longer and the apical cell is more rounded than in *M. spicata*. The length:width ratio of *M. cardiaca* leaf is 1.85 while that ratio of *M. spicata* is 2.5.

The Volatile Oil

The volatile oil was collected in two portions: the oil, separated by distillation, and the cohobated oil. The oil was a red-brown oil with a sweet spearmint-like odor. The cohobated oil was amber colored and possessed a similar odor. The latter oil made up 8% of the total oil yield.

Physical constants.—Oil: Sp. gr. $^{25}_{25}$ 0.9128; $[\alpha]^{25}_{D}$ −69.85; n^{20}_{D} 1.4871. Cohobated Oil: Sp. gr. $^{25}_{25}$ 0.944; $[\alpha]^{25}_{D}$ −56.91; n^{20}_{D} 1.4925.

Ketones.—The neutral sulfite method (1) gave 57% average for the oil and 84% average for the cohobated oil. The absorption product of each was decomposed and the oils collected by steam distillation. Found: n^{20}_{D} 1.498, corresponding to carvone, for each oil. The oxime prepared from each oil had a melting point of 72°, corresponding to carvone.

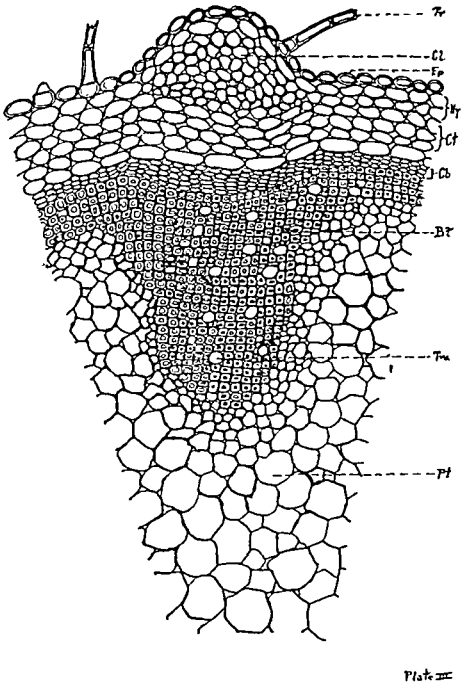

Fig. 3.—*Mentha cardiaca* Gerarde. T. S. of stem. *Tr*, trichome; *Cl*, collenchyma; *Ep*, epidermis; *Hy*, hypodermis; *Cl*, cortical parenchyma; *Cb*, cambium; *Bf*, fibers; *Tra*, trachea; *Pt*, pith.

Acid Number.—The method of Guenther (2) gave an average of 0.556 for the oil and 1.005 for the cohobated oil.

Esters.—The ester number for the oils was determined according to the method of Guenther (3). Results were variable. The results of eight determinations with each oil gave values between 12.64 and 19.79 for the oil and between 14.73 and 23.68 for the cohobated oil. An average of 16.215 for the oil corresponds to 5.6% of linalyl acetate. A similar calculation gave 6.7% for the cohobated oil.

Alcohols.—The ester number after acetylation (4) was 28.82 for the oil and 42.97 for the cohobated oil. By using the average ester values and these results, the free alcohol, calculated as linalool, was found to be 3.5% in the oil and 6.6% in the cohobated oil.

Phenols.—The general procedure of Guenther (5) demonstrated an absence of phenolic bodies in both oils.

SUMMARY

A histological study of the leaf and stem of *Mentha cardiaca* Gerarde has been presented. A comparison with *M. spicata*, of typical structures, was made.

The growth characteristics, crop yield data, and oil yield of a one-year-old planting have been recorded.

A partial chemical investigation of the volatile oil has been made. Data are reported for certain physical and chemical constants. The distilled oils contain a high ketone content, the identity of which is carvone.

Since this is a preliminary investigation of the volatile oil of *Mentha cardiaca* Gerarde, additional investigation will be necessary before a complete report can be made.

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The Properties of the Oil of *Xanthium Spinosum**

By C. JELLEFF CARR

Some chemical and physical properties of the oil obtained from *Xanthium spinosum* are reported. Preliminary studies on toxicity and on blood pressure response indicate that the oil is pharmacodynamically inert.

XANTHIUM, cocklebur or spiny clotbur is a member of the *Composita* family naturalized in this country from Europe. The burs of the plant have been employed in the preparation of a tea infusion for use by mouth in the treatment of arthralgias. Clinical and pharmacologic investigations have been conducted with extracts of this plant. Previously, the chemistry and pharmacology of *Xanthium spinosum* was reported in THIS JOURNAL (1). Choline was isolated from aqueous extracts prepared from the burs of the plant by forming the readily crystallizable perchlorate salt of the nitrate ester (2). During the preparation of aqueous extracts of

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xanthium burs it was observed that an oil separated from the aqueous concentrates. Subsequently the burs were rendered fat-free by repeated extraction with petroleum ether. The pale yellow oil obtained in this manner was subjected to a chemical and pharmacologic examination.

A glycoside and an unidentified active principle have been obtained from *Xanthium strumarium* (3), and Maksimov obtained an oil from the seeds of this plant and described its physical and chemical properties as resembling those of sunflower-seed oil (4).

The burs used in this investigation were identified pharmacognostically as *Xanthium spinosum* by Professor Heber W. Youngken. The oil was obtained from burs from plants grown in Iowa, Maryland, and Ohio.

EXPERIMENTAL

Isolation of the Oil.—The air-dried, coarsely ground burs were extracted in a percolator with petroleum ether until the drug was exhausted. The percolate was collected and the petroleum ether recovered by distillation on a steam bath. A light yellow oil was recovered. The average oil yield was 7.9% of the weight of the air-dried burs. A fine crystalline material separated from the oil, which was identified as stearin (m. p. 65°) and an unidentified wax (m. p. 83°). The oil was dried for one month in a desiccator under a vacuum at room temperature and subjected to the following examination.

Description of the Oil.—The dry, clear, light yellow oil obtained by the method previously described exhibits a peculiar characteristic odor. It is tasteless and burns with a smoky flame and with a typical fatty odor. The oil is acid to moist litmus paper. It is insoluble in alcohol and slightly soluble in boiling alcohol. Xanthium oil is very soluble in chloroform and acetone, and soluble to a limited degree in isopropanol. It is slightly soluble in boiling water.

Three oils prepared from different samples of the drug, from which the stearin had been removed, gave the following refractometer readings: n_D^{25} 1.4750, n_D^{20} 1.4755, and n_D^{20} 1.4755. The specific gravity at 28° was 0.920.

Xanthium oil when viewed in an ultraviolet light with a Wood's filter exhibits a strong purple-blue fluorescence differing from cottonseed oil, which exhibits a lighter blue fluorescence.

Xanthium oil prepared from different samples of the burs gave the following values for fats and fatty oils as determined by the methods described in United States Pharmacopœia XIII. The free fatty acids in xanthium oil gave an acid value of 20.2 cc. of 0.1 *N* alkali required to neutralize the free acid in 10 Gm. of the oil. The saponification value was found to be approximately 200. An average ester value of 188 was obtained with an iodine value of 130. A light yellow wax constituting approximately 3.5% of the oil was obtained as unsaponifiable matter. This wax became hard and brittle upon aging. The soap prepared from the saponified oil possessed good cleansing properties. The esters of the fatty acids present in the oil are glyceryl esters. A satisfactory yield of glycerin was obtained from one sample of the oil.

After separation of the stearin and wax by cooling, the oil was distilled under a reduced pressure of approximately 30 mm. Hg. A trace of a volatile fraction was obtained between 20–100° and approximately equal volumes of three fractions distilling between 100–240° and 240–360°, and a heavy oil residue that does not solidify at room temperature. There was very little decomposition of the oil during distillation at these temperatures. Samples of the oil exposed in a desiccator to sunlight and air for six years have retained their pale yellow color and have not developed a rancid odor. A thin film of gum formed upon the surface of the oil.

The mixed fatty acids prepared from the oil according to the U. S. P. method are dark brown in color and do not solidify at room temperature. At 15° the liquid mixed fatty acids begin to solidify and at 5° are almost completely solid. For this reason not more than traces of palmitic or stearic acids are believed to be present.

Miscellaneous Tests.—An examination of the oil for vitamin A by the Carr-Price reaction gave a negative test. The U. S. P. identity test for cottonseed oil with a solution of sulfur in carbon disulfide and amyl alcohol yields with xanthium oil a brown color in place of the brilliant red of cottonseed oil. A negative test for resin oils was obtained. Baudouin's test yields a deep red color with xanthium oil. The color resembles that produced by sesame oil.

The oil was found to be neither attractive nor repelling to a variety of insects when applied to the skin and when substituted for the oil employed as a lure in insect traps.

Pharmacodynamic Activity.—In experiments to determine the nature of the depressor substance in xanthium the following studies were made. The intravenous injection in the dog of from 2 to 5 cc. of physiological salt solution saturated with xanthium oil did not significantly influence the blood pressure. The injection of from 2 to 5 cc. of a 10% solution of the polyols obtained from the saponification of the oil produced a fall in blood pressure of from 10 to 22 mm. of Hg. (The injection of an equivalent dose of glycerin was without effect upon the blood pressure.) The saponified oil, upon intravenous injection of 2 cc. of a 5% solution produced a fall in blood pressure equivalent to that elicited by an equivalent dose of sodium oleate. Saturated aqueous solutions of the mixed fatty acids obtained from xanthium oil were without effect upon the blood pressure when injected intravenously. A 5% emulsion of the oil upon intravenous injection elicited no depressor response.

The feeding of a diet containing 1% of xanthium oil to white rats for a period of 6 weeks produced no visible toxic effects. The animals gained weight at a normal rate. The oral administration of doses of from 1 to 2 cc. of xanthium oil by stomach tube to white rats produced only a mild degree of catharsis but no toxic symptoms during an observation period of two days.

From the animal experiments it appears that the fixed oil obtained from *Xanthium spinosum* is

relatively pharmacologically inert and probably does not represent a therapeutically active portion of the plant.

SUMMARY

The properties of the oil obtained from the burs of *Xanthium spinosum* have been described. Preliminary studies on blood pressure response and on toxicity indicate that the oil is pharmacodynamically inert.

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Radio-Frequency Drying of Tablet Granulations*

By H. BIKIN, GLENN L. JENKINS and H. GEORGE DeKAY

This project was undertaken to survey the possibilities of utilizing radio-frequency drying of tablet granulations for future work of a more extensive nature. Two possible theories concerning the actual mechanism of nonconductor materials in a radio-frequency field are reviewed explaining how the nonconductor material becomes heated from within rather than by application of heat to its perimeters and the subsequent transfer of the heat to the center. A number of important electrical considerations regarding the size and materials of construction of the electrodes and their positions relative to one another are discussed. A table of thirty-one chemicals, with the percentage of added water removed, the period of drying time, and the peak radio-frequency voltage used, is given.

THE PROCESS of manufacturing tablets can be broken down roughly into the following steps: weighing, mixing, granulating, drying, and compressing. If the drying operation, which is very time-consuming, could be handled by radio-frequency radiation, the manufacturing cycle could be multiplied by a factor and a continuous production line would become feasible.

Drying consists of two phases: the separation of the media to be eliminated from the material to be dried, and the actual removal of the separated media from the material. Only the first phase can be accelerated to give an appreciable decrease in over-all drying time by increasing the amount of heat applied to the material during the drying operation.

Radio-frequency radiation drying makes use of the heating effect caused in nonconductor materials by the rapid alternations of the radio-frequency field. In addition to placing the material between two parallel plate electrodes to effect drying, it is also possible to use the stray field method in which the electrodes consist of a series of parallel rods or tubes over which the material passes. Alternate electrodes are connected together with one set tied to the high voltage source and the other set to the ground lead to form an electrical field of an ellipsoidal shape between them. At present, either of the two following concepts, concerning what actually takes place in a material which is placed in a radio, frequency field, are acceptable.

According to Duryee (1), each substance is composed of a positively charged nucleus around which negative electrons revolve in fixed orbits with the number of orbits dependent upon the atomic structure of the substance. As this substance is subjected to a high-voltage, high-frequency field, the orbits of electron travel are severely stressed and cause molecular friction. This friction results in heat generation and the whole mass of the substance becomes uniformly heated from within.

The explanation given by Venable (2) states that with no external electrical field present, a molecule might be seen to have its centers of positive and negative charges coincident, or these centers may be displaced relative to one another. As the external electrical field changes, the molecule described in the former instance might be seen to have its coincident centers displaced relative to one another forming individual dipoles whose axes tend to parallel the electrical field. The molecule described in the latter instance is called a permanent molecular dipole and it too

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will tend to rotate until its axis is parallel to the electrical field. In some cases there also may be an added displacement of the electrical centers of the permanent dipoles. Work is done by the displacement of the electrical centers of the molecules, by the rotation of the dipoles, or by both.

Materials which are being dried do not begin to heat up excessively until the point of latent heat of vaporization has been passed, and if, at that time, they can instantly be removed from the drying chamber there will be no perceptible rise in temperature (3). In ordinary drying methods this situation is very difficult to control because the heat is first applied to the perimeters of the material and then is transferred to the center. In radio-frequency drying the heat is generated within the substance so that the entire mass is uniformly heated and additional heat can be discontinued at any second by throwing a switch and breaking a circuit.

Actual experimental work with a radio-frequency drying unit must be preceded by a correlation of the limiting electrical requirements of this new tool. To obtain a uniform field, the electrodes must necessarily be equidistant at all points. Large electrodes may cause nonuniform heating due to standing waves (4), but this may be overcome by "multiple tuning"[1] or by reducing the dimensions of the electrodes to less than one-eighth of a wave length of the frequency being used (5). Brass, steel, copper, or aluminum electrodes show no appreciable differences although aluminum may be preferred because of its weight and its resistivity to corrosion. All sharp corners should be removed in order to prevent corona arcing and to increase the amount of permissible voltage to be applied. The dimensions of the parallel plate electrodes should exceed those of the material placed in the radio-frequency field to keep the material out of the uneven fringed field which forms around the edges of the electrodes. To prevent the moisture given off by the granules from condensing upon the upper electrodes and to aid in the removal of the moisture separated from the granules, a small fan is useful.

Each of the chemicals in Table I was mixed with the amount of distilled water deemed necessary to obtain a granule when the material was passed through a 30-mesh sieve. The percentage of moisture lost was based upon the amount of water added to the granulation.

TABLE I.—DRYING OF CHEMICALS

Chemical	Grams Used	Grams of H_2O Added	Kv. (Peak R.F.)	Time, Min.	% H_2O Removed
Aspirin	194.0	31.0	8.0	15	95.3
Starch	117.1	30.4	10.4	30	84.0
Sulfanilamide	133.5	35.5	8.5	30	100.0
Acetanilid	137.1	21.9	8.75	45	89.2
Acetophenetidin	178.4	28.6	8.5	45	91.0
Sodium Chloride	253.5	13.4	12.5	30	100.5
Sucrose	183.2	9.3	11.0	30	102.2
Calcium Gluconate	94.1	26.4	8.5	30	96.8
Calcium Carbonate	144.0	48.0	8.5	45	95.8
Magnesium Carbonate	72.5	79.5	8.0	30	105.0
Dextrin	128.0	20.5	9.0	30	98.5
Ammonium Chloride	128.6	45.9	13.0	135	94.0
Sulfathiazole	162.4	21.1	8.0	30	94.7
Sodium Nitrate	324.5	8.5	9.0	45	71.0
Sodium Borate	130.8	32.7	9.0	45	98.0
Tribasic Sodium Phosphate	102.5	7.0	9.5	45	107.0
Calcium Sulfate	165.2	48.8	8.0	30	74.0
Tartaric Acid	128.9	8.6	9.0	15	93.0
Tribasic Magnesium Phosphate	42.0	100.0	9.5	105	73.0
Magnesium Trisilicate	124.0	31.0	8.0	15	111.0
Tribasic Calcium Phosphate	213.1	25.4	8.0	15	97.8
Dicalcium Phosphate	221.0	26.5	8.0	15	98.0
Sodium Acid Phosphate	185.6	22.4	12.0	45	40.1
Magnesium Oxide	98.3	59.7	8.0	45	80.2
Dextrose	175.2	10.8	12.0	60	102.0
Calcium Lactate	158.3	31.7	6.5	45	91.5
Sodium Sulfate	218.9	18.6	9.0	45	89.0
Methenamine	146.2	12.4	9.0	45	80.6
Aminopyrine	161.3	28.2	8.0	45	89.0
Bismuth Subcarbonate	147.5	34.0	13.0	45	95.7
Phenobarbital	123.8	40.7	8.5	30	95.7

[1] R. A. Bierwirth, U. S. Patent 2308013 (1943)

While aspirin, dried by radio-frequency heating, does hydrolize to some extent as evidenced by the pronounced odor of acetic acid, nevertheless the free salicylic acid test of the United States Pharmacopœia is not exceeded.

Sodium bicarbonate loses over ninety per cent of the added moisture when dried in this manner but it decomposes so that the carbonate test of the United States Pharmacopœia is exceeded.

Of outstanding importance is the fact that a good number of widely used chemicals and tablet excipients can be dried rapidly and easily.

SUMMARY

Of the thirty-one chemicals chosen, twenty-seven were dried of eighty per cent or more of the added moisture in a satisfactory manner.

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Infrared Drying of Tablet Granulations. I*

By B. N. PATEL, GLENN L. JENKINS and H. GEORGE DeKAY

Recent improvements in the manufacture of compressed tablets have been outlined and the need for a rapid and continuous drying procedure for tablet granulations indicated. Infrared drying equipment is discussed and the possible advantages of such procedures are presented. The results of the experiments carried out in this work lead to the conclusion that infrared drying is rapid and uniform and could be used advantageously in drying tablet granulations.

NUMEROUS medicinal agents have been administered in the form of compressed tablets for the past few decades. It has been estimated that close to one-third of the medication used today is administered in this form.

The manufacture of compressed tablets involves four important steps: the weighing of the ingredients, the granulating, the drying, and the compressing of the dried granulation into desired shapes of tablets. Much progress has been made in the field of tablet manufacture in recent years through the use of perfected machinery. Mixing, granulating, and compressing have been improved to such an extent that large quantities can be processed in relatively short periods of time.

The operation of drying the granulation has remained practically unchanged and it requires at present as much time, if not more, as the weighing, granulating, and compressing combined. Many types of drying closets are used. For years (1) the most popular was the ordinary closet, arranged in series, heated by steam coils in the back, and having an opening near the base and an opening at the top for the escape of evaporated moisture. Later came developments involving the use of the air in outside chambers and the uniform distribution of the hot air by means of properly arranged flues (1).

Cook and LaWall (2) state that a number of laboratories manufacturing a line of tablets are provided with special equipment which automatically controls the humidity and temperature of the whole laboratory space in which the processes of tabletting are executed. This points out how essential proper drying facilities are considered to be by experienced tablet makers.

According to Chilson (5), drying signifies a change in the vapor pressure of the moisture contained in a solid. Since this change must be produced by heat, all apparatus designed for drying or dehydration is simply a means of conveying heat to the moisture contained in a solid. Thus to shorten the time needed to dry tablet granulations, devices must be found which will transfer more heat to the moisture in the granulation than the dry warm air or an electric oven, and which will promote more uniform heating.

Victor (4) states that good results have been obtained by using low drying temperatures in an oven with controlled humidity. The majority of granulations can be dried efficiently to the required final moisture content at relatively high humidities and at moderate temperatures.

The trend in the use of tablets has been one of steady increase. The National Formulary pioneered in the establishment of official standards for tablets. Tablets increased (6) virtually

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from none in 1920 to 17 per cent of all the official admissions of dosage forms in 1945. This expansion in the field of the manufacture of compressed tablets calls for a method or methods whereby the drying time could be sufficiently shortened. It should also be essential to design equipment for a continuous process which would take at most not more than two or three hours from the time the powders were weighed until the first tablets were compressed.

The various methods (5) of drying are: (1) convection drying (2) radiation drying, (3) conduction drying, (4) induction drying, (5) electronic drying, (6) resistance drying, (7) absorption drying, and (8) sublimation drying. Of the various ways of applying heat to an object, radiation is recognized as the most efficient, provided the path that the radiant energy has to travel can be kept short (3). It is characteristic of this type of drying that heat will be radiated from a heat source regardless of interposed air. It is, therefore, the object of this research project to investigate the possibility of using heat radiations produced by infrared lamps to shorten the time required to dry tablet granulations without adverse effects on the materials treated.

EXPERIMENTAL

Lamps encased in a metal casing as supplied by The Fostoria Pressed Steel Corporation, Fostoria, Ohio, were mounted on a wooden rack in a stable position. The wooden rack was supported by screwed supports which can be replaced by shorter or longer supports as required to decrease or increase the distance between the material heated and the source of heat. The material to be dried was placed in a suitable container directly below the lamps.

A. **Heating of Chemicals.**—To determine whether the heat from the lamps would cause any decomposition of chemicals commonly used in tablet making, a number of chemicals were heated in 5-Gm. quantities in Petri dishes or paper dishes for twenty minutes under 250-watt, 375-watt (blue), and 500-watt bulbs at distances of 1 foot and 2 feet. Visual physical changes, if any, were noted and changes in weight determined.

The results of the experiment are depicted in Table I.

It will be observed from the table that a majority of the substances do not undergo any changes when thus heated under lamps. The temperature reached during twenty minutes' heating ranged from 70° to 75° in the case of the 250-watt white bulb, 95° to 115° in the case of the 375-watt blue bulb, and 95° to 100° in the case of the 500-watt white bulb when the distance was 1 foot. Corresponding temperatures at a distance of 2 feet are 45° to 50°, 83° to 85°, and 55° to 58°.

B. **Drying Tablet Granulations.**—Various tablet granulations prepared according to known formulas using starch paste as a granulating agent, unless otherwise stated, were dried under different lamps at a distance of 22 inches and with a layer thickness of 1/4 inch. (The starch paste used was prepared as follows: mix one part of corn starch with three parts of cold water in a beaker, place the mixture in a boiling water bath and stir until the paste begins to thicken. Remove the beaker and cool in cold water with continuous stirring.) The dried granulations were compressed after proper lubrication. The results of this experiment are given in Table II.

C. **Prolonged Heating of Chemicals.**—In order to ascertain the effects of prolonged heating by infrared lamps on chemicals, a few of the chemicals used in granulations during this work were heated for two hours under 375-watt and 500-watt lamps, keeping the distance at 22 inches; then the loss in weight was determined.

Among the chemicals thus treated, the following showed no apparent changes: acetanilid, aspirin, aminopyrine, calcium lactate, calcium phosphate, lactose, phenacetin, potassium bicarbonate, potassium bromide, sulfanilamide, sulfathiazole, sodium benzoate, and sodium salicylate. Amytal, bismuth subnitrate, caffeine citrate, lithium citrate, and sulfapyridine showed slight loss in weight while magnesium trisilicate showed considerable loss in weight when heated under a 500-watt bulb and benzocaine underwent decomposition under 375 watt and 500-watt bulbs.

CONCLUSIONS

From the work done under the present project the following conclusions were drawn:

1. Heating by infrared lamps was rapid and the heat distribution was uniform throughout. Best results were obtained with a 375-watt white bulb at a distance of 22 inches. The 375-watt blue bulb caused decomposition in many cases. It was not clear whether the chemicals which were designated as light-sensitive (of course, to different extents) were affected by the infrared light or not.

2. Rate of drying depends, as expected, on the moisture content of the material and the thickness of the bed of granules. Thickness of 1/4 inch gave better results than thickness of 1/2 inch.

3. Use of radiant heating by infrared lamps on an industrial scale can be more economical as compared to air-drying ovens since, in the latter case, it is necessary in big factories to maintain the oven constantly at heat in readiness for work at all times. Further, it is necessary that the temperature be brought to a peak in the ovens long enough before they are to be used so that they will be ready to dry the material put into them. For the same reasons, a substantial amount of stored heat is lost at the end of the day if the ovens are maintained on an eight- or sixteen-hour cycle. With the infrared equipment it is not necessary to turn on the light until the granulation is actually ready for drying.

TABLE I.—EFFECTS OF INFRARED HEAT ON CHEMICALS

No.	Material	250-Watt White Bulb 1 Ft.	250-Watt White Bulb 2 Ft.	375-Watt Blue Bulb 1 Ft.	375-Watt Blue Bulb 2 Ft.	500-Watt White Bulb 1 Ft.	500-Watt White Bulb 2 Ft.
1	Acetanilide	...	NC		LD	..	NC
2	Aloin	...	NC		B	...	SL
3	Aminopyrine	NC	NC	LD	NC	LD	NC
4	Ammonium Bromide	...	NC	.	NC	...	NC
5	Ammonium Chloride	...	NC	...	NC	..	NC
6	Amytal	...	NC	.	NC	.	NC
7	Arsenic Trioxide	...	NC		NC	...	SL
8	Ascorbic Acid	NC	NC	B	B	NC	NC
9	Benzocaine	D	NC	LD	LD	LD	NC
10	Benzoic Acid	...	NC	...	LD	..	D
11	Bismuth Subcarbonate	...	NC	..	NC	.	NC
12	Bismuth Subgallate	...	NC	...	SL		NC
13	Bismuth Subnitrate	...	NC	.	SL	.	NC
14	Borax	...	NC	.	NC	..	NC
15	Boric Acid	...	NC		SL		NC
16	Caffeine	NC	NC	NC	NC	SL	NC
17	Caffeine Citrate	...	NC	..	NC		NC
18	Calcium Bromide	...	NC	..	NC	.	NC
19	Calcium Carbonate	...	NC		NC		NC
20	Calcium Chloride	...	W	..	W	.	W
21	Calcium Gluconate	NC	NC	NC	NC	NC	NC
22	Calcium Glycerophosphate	...	NC	.	SL	...	NC
23	Calcium Lactate	NC	NC	SL	SL	SL	SL
24	Calcium Phosphate (dibasic)	...	NC		NC	..	NC
25	Calcium Sulfide	...	NC	.	NC	.	NC
26	Camphor (monobromated)	...	NC	.	LD	...	LD
27	Cerium Oxalate	...	NC	...	SL	..	NC
28	Chloramine T	...	D	...	D	...	D
29	Cincophen	...	NC	..	NC	...	NC
30	Dextrin	NC	SL	SL	SL	SL	SL
31	Dextrose	...	NC	.	NC	...	NC
32	Ephedrine (Alkaloid)	LD	LD	LD	LD	LD	LD
33	Ephedrine Sulfate	NC	NC	NC	NC	NC	NC
34	Ferrous Sulfate (dried)	...	NC	..	NC	...	NC
35	Gelatin	SL	SL	SL	SL	SL	SL
36	Lactose	NC	NC	NC	NC	NC	NC
37	Lithium Citrate	...	NC		SL	..	NC
38	Magnesium Carbonate	...	NC	..	NC	.	SL
39	Magnesium Oxide (heavy)	...	SL		SL		NC
40	Magnesium Salicylate	...	NC		D		NC
41	Magnesium Sulfate	...	NC		D	..	NC
42	Magnesium Trisilicate	...	SL	..	SL	..	SL
43	Mercuric Iodide	...	NC	..	SL	..	SL
44	Mercurous Chloride	...	NC	..	W	...	NC
45	Mercurous Cyanide	...	NC		NC		NC
46	Methenamine	...	NC	.	NC		NC
47	Pepsin	...	SL	.	SL	...	SL
48	Phenacetin	NC	NC	D	NC	NC	NC
49	Phenolphthalein	...	NC	.	NC	..	NC
50	Potassium Bicarbonate	...	NC	.	NC	..	NC
51	Potassium Chloride	..	NC	.	NC	..	NC
52	Potassium Citrate	.	NC		NC	..	NC
53	Salicylic Acid	..	NC		D	..	NC
54	Salol	...	LD		LD	.	LD
55	Sodium Benzoate	.	SL	.	NC		SL
56	Sodium Bicarbonate	SL	NC	SL	NC	SL	NC
57	Sodium Bromide	...	NC	..	W	.	NC
58	Sodium Chloride	...	NC	..	NC	..	NC
59	Sodium Citrate	..	NC	...	NC	...	NC
60	Sodium Nitrate	...	NC	...	NC	...	NC
61	Sodium Salicylate	...	NC	...	NC	...	NC
62	Starch (potato)	SL	SL	SL	SL	SL	SL
63	Sulfanilamide	NC	NC	NC	NC	NC	NC
64	Sulfapyridine	NC	NC	NC	NC	NC	NC
65	Sulfathiazole	NC	NC	NC	NC	NC	NC
66	Tartar Emetic	...	NC	...	NC	...	NC
67	Zinc Phosphide	...	NC	...	NC	...	NC

The letters used in the table will be interpreted as follows: D = decomposition; W = mass becoming damp; SL = slight loss in weight; B = darkening; NC = no apparent change; LD = liquefaction and decomposition.

from none in 1920 to 17 per cent of all the official admissions of dosage forms in 1945. This expansion in the field of the manufacture of compressed tablets calls for a method or methods whereby the drying time could be sufficiently shortened. It should also be essential to design equipment for a continuous process which would take at most not more than two or three hours from the time the powders were weighed until the first tablets were compressed.

The various methods (5) of drying are: (1) convection drying (2) radiation drying, (3) conduction drying, (4) induction drying, (5) electronic drying, (6) resistance drying, (7) absorption drying, and (8) sublimation drying. Of the various ways of applying heat to an object, radiation is recognized as the most efficient, provided the path that the radiant energy has to travel can be kept short (3). It is characteristic of this type of drying that heat will be radiated from a heat source regardless of interposed air. It is, therefore, the object of this research project to investigate the possibility of using heat radiations produced by infrared lamps to shorten the time required to dry tablet granulations without adverse effects on the materials treated.

EXPERIMENTAL

Lamps encased in a metal casing as supplied by The Fostoria Pressed Steel Corporation, Fostoria, Ohio, were mounted on a wooden rack in a stable position. The wooden rack was supported by screwed supports which can be replaced by shorter or longer supports as required to decrease or increase the distance between the material heated and the source of heat. The material to be dried was placed in a suitable container directly below the lamps.

A. Heating of Chemicals.—To determine whether the heat from the lamps would cause any decomposition of chemicals commonly used in tablet making, a number of chemicals were heated in 5-Gm. quantities in Petri dishes or paper dishes for twenty minutes under 250-watt, 375-watt (blue), and 500-watt bulbs at distances of 1 foot and 2 feet. Visual physical changes, if any, were noted and changes in weight determined.

The results of the experiment are depicted in Table I.

It will be observed from the table that a majority of the substances do not undergo any changes when thus heated under lamps. The temperature reached during twenty minutes' heating ranged from 70° to 75° in the case of the 250-watt white bulb, 95° to 115° in the case of the 375-watt blue bulb, and 95° to 100° in the case of the 500-watt white bulb when the distance was 1 foot. Corresponding temperatures at a distance of 2 feet are 45° to 50°, 83° to 85°, and 55° to 58°.

B. Drying Tablet Granulations.—Various tablet granulations prepared according to known formulas using starch paste as a granulating agent, unless otherwise stated, were dried under different lamps at a distance of 22 inches and with a layer thickness of 1/4 inch. (The starch paste used was prepared as follows: mix one part of corn starch with three parts of cold water in a beaker, place the mixture in a boiling water bath and stir until the paste begins to thicken. Remove the beaker and cool in cold water with continuous stirring.) The dried granulations were compressed after proper lubrication. The results of this experiment are given in Table II.

C. Prolonged Heating of Chemicals.—In order to ascertain the effects of prolonged heating by infrared lamps on chemicals, a few of the chemicals used in granulations during this work were heated for two hours under 375-watt and 500-watt lamps, keeping the distance at 22 inches; then the loss in weight was determined.

Among the chemicals thus treated, the following showed no apparent changes: acetanilid, aspirin, aminopyrine, calcium lactate, calcium phosphate, lactose, phenacetin, potassium bicarbonate, potassium bromide, sulfanilamide, sulfathiazole, sodium benzoate, and sodium salicylate. Amytal, bismuth subnitrate, caffeine citrate, lithium citrate, and sulfapyridine showed slight loss in weight while magnesium trisilicate showed considerable loss in weight when heated under a 500-watt bulb and benzocaine underwent decomposition under 375 watt and 500-watt bulbs.

CONCLUSIONS

From the work done under the present project the following conclusions were drawn:

1. Heating by infrared lamps was rapid and the heat distribution was uniform throughout. Best results were obtained with a 375-watt white bulb at a distance of 22 inches. The 375-watt blue bulb caused decomposition in many cases. It was not clear whether the chemicals which were designated as light-sensitive (of course, to different extents) were affected by the infrared light or not.

2. Rate of drying depends, as expected, on the moisture content of the material and the thickness of the bed of granules. Thickness of 1/4 inch gave better results than thickness of 1/2 inch.

3. Use of radiant heating by infrared lamps on an industrial scale can be more economical as compared to air-drying ovens since, in the latter case, it is necessary in big factories to maintain the oven constantly at heat in readiness for work at all times. Further, it is necessary that the temperature be brought to a peak in the ovens long enough before they are to be used so that they will be ready to dry the material put into them. For the same reasons, a substantial amount of stored heat is lost at the end of the day if the ovens are maintained on an eight- or sixteen-hour cycle. With the infrared equipment it is not necessary to turn on the light until the granulation is actually ready for drying.

TABLE I.—EFFECTS OF INFRARED HEAT ON CHEMICALS

No.	Material	250-Watt White Bulb 1 Ft.	250-Watt White Bulb 2 Ft.	375-Watt Blue Bulb 1 Ft.	375-Watt Blue Bulb 2 Ft.	500-Watt White Bulb 1 Ft.	500-Watt White Bulb 2 Ft.
1	Acetanilide	...	NC		LD	...	NC
2	Aloin	...	NC		B	...	SL
3	Aminopyrine	NC	NC	LD	NC	LD	NC
4	Ammonium Bromide	...	NC	.	NC	...	NC
5	Ammonium Chloride	...	NC	..	NC	.	NC
6	Amytal	...	NC		NC	..	NC
7	Arsenic Trioxide	...	NC		NC	...	SL
8	Ascorbic Acid	NC	NC	B	B	NC	NC
9	Benzocaine	D	NC	LD	LD	LD	NC
10	Benzoic Acid	...	NC	...	LD	..	D
11	Bismuth Subcarbonate	...	NC	...	NC	.	NC
12	Bismuth Subgallate	...	NC	..	SL		NC
13	Bismuth Subnitrate	...	NC	.	SL		NC
14	Borax	...	NC		NC	...	NC
15	Boric Acid	...	NC		SL		NC
16	Caffeine	NC	NC	NC	NC	SL	NC
17	Caffeine Citrate	...	NC	...	NC	.	NC
18	Calcium Bromide	...	NC	.	NC	.	NC
19	Calcium Carbonate	...	NC		NC	.	NC
20	Calcium Chloride	...	W	...	W	..	W
21	Calcium Gluconate	NC	NC	NC	NC	NC	NC
22	Calcium Glycerophosphate	...	NC		SL	...	NC
23	Calcium Lactate	NC	NC	SL	SL	SL	SL
24	Calcium Phosphate (dibasic)	...	NC		NC	..	NC
25	Calcium Sulfide	...	NC		NC	.	NC
26	Camphor (monobromated)	...	NC	.	LD	..	LD
27	Cerium Oxalate	...	NC	..	SL	..	NC
28	Chloramine T	...	D	...	D	...	D
29	Cincophen	...	NC	...	NC	..	NC
30	Dextrin	NC	SL	SL	SL	SL	SL
31	Dextrose	...	NC		NC	...	NC
32	Ephedrine (Alkaloid)	LD	LD	LD	LD	LD	LD
33	Ephedrine Sulfate	NC	NC	NC	NC	NC	NC
34	Ferrous Sulfate (dried)	...	NC	.	NC	...	NC
35	Gelatin	SL	SL	SL	SL	SL	SL
36	Lactose	NC	NC	NC	NC	NC	NC
37	Lithium Citrate	...	NC		SL	..	NC
38	Magnesium Carbonate	...	NC	...	NC		SL
39	Magnesium Oxide (heavy)	...	SL	.	SL		NC
40	Magnesium Salicylate	...	NC		D		NC
41	Magnesium Sulfate	...	NC		D	..	NC
42	Magnesium Trisilicate	...	SL		SL	.	SL
43	Mercuric Iodide	...	NC	.	SL	.	SL
44	Mercurous Chloride	...	NC		W	...	NC
45	Mercurous Cyanide	...	NC		NC		NC
46	Methenamine	...	NC		NC		NC
47	Pepsin	...	SL	.	SL	...	SL
48	Phenacetin	NC	NC	D	NC	NC	NC
49	Phenolphthalein	..	NC	.	NC	..	NC
50	Potassium Bicarbonate	..	NC		NC	..	NC
51	Potassium Chloride	..	NC	..	NC	..	NC
52	Potassium Citrate	.	NC		NC	..	NC
53	Salicylic Acid	..	NC		D	..	NC
54	Salol	..	LD		LD	.	LD
55	Sodium Benzoate	.	SL		NC	.	SL
56	Sodium Bicarbonate	SL	NC	SL	NC	SL	NC
57	Sodium Bromide	...	NC	..	W	.	NC
58	Sodium Chloride	.	NC	..	NC	...	NC
59	Sodium Citrate	..	NC	...	NC	...	NC
60	Sodium Nitrate	...	NC	...	NC	...	NC
61	Sodium Salicylate	...	NC	...	NC	...	NC
62	Starch (potato)	SL	SL	SL	SL	SL	SL
63	Sulfanilamide	NC	NC	NC	NC	NC	NC
64	Sulfapyridine	NC	NC	NC	NC	NC	NC
65	Sulfathiazole	NC	NC	NC	NC	NC	NC
66	Tartar Emetic	...	NC	...	NC	...	NC
67	Zinc Phosphide	...	NC	...	NC	...	NC

The letters used in the table will be interpreted as follows: D = decomposition; W = mass becoming damp; SL = slight loss in weight; B = darkening; NC = no apparent change; LD = liquefaction and decomposition.

TABLE II.—COMPRESSION RESULTS ON DRIED GRANULATIONS

Granulation	Bulb,[a] Watt	Results of Drying	Time Required, Min.	Compression
Bismuth Subnitrate	250	Incomplete[b]	80	
Calcium Lactate	250	Dried	101	Satisfactory
Alkaline, Aromatic	500	Dried	40	Satisfactory
Aspirin Compound	500	Dried	40	Satisfactory
Alkaline, Effervescent	500	Dried	35	Satisfactory
Antiseptic	500	Decomposition		
Acetanilid & Caffeine Compound	250	Dried (Loss of Camphor)	70	Satisfactory
Acetanilid & Caffeine	500	Dried	40	Satisfactory
Sulfanilamide	375	Dried	55	Satisfactory
Sodium Bromide Compound	375	Dried	60	Satisfactory
Aminopyrine (Starch paste)	375	Decomposition		
Aminopyrine (Gelatin-acacia solution)	375	Dried	50	Capping
Sulfathiazole	375	Dried	50	Satisfactory
Mixed Treatment #2	375	Dried	80	Satisfactory
Phenacetin & Salol	250	Liquefied		
	375	Liquefied		
Benzocaine Compound	250	Dried	55	Satisfactory
	375	Dried	55	Satisfactory

[a] Two bulbs were used to dry the material. They were white bulbs.
[b] The wet granulation dried completely on 15 minutes' additional heating under two 500-watt bulbs.

4. There is a field for further study in the drying of tablet granulations by infrared lamps. Granulating equipment can be connected to a drying tunnel consisting of a movable belt or screen as a means of conveying the uniformly spread wet granules to be dried by the heat of the infrared lamps attached to the inner walls of the tunnel; thus granulation can be effected continuously. The tunnel can be constructed on the principles of the automobile industry which uses infrared lamps to bake paints on automobile bodies. The tunnels can be equipped so as to process conveniently small as well as large batches.

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Infrared Drying of Tablet Granulations. II*

By B. N. PATEL, GLENN L. JENKINS and H. GEORGE DeKAY

Twenty-two formulas containing inorganic compounds, nineteen formulas containing organic compounds, and fourteen compound formulas were granulated using the usual technique. The granules were dried under infrared lamps and compressed after lubrication. Favorable results were obtained in about ninety per cent of the cases. Temperature and period of drying in a majority of the cases were below 60° and 25 minutes, respectively. Results of assays on the granulations showed complete absence of untoward effects of infrared heat.

THIS IS A continuation of the previous work (29) undertaken to investigate the possibility of using infrared lamps for successfully drying tablet granulations. The nature of the problem has been outlined in the previous paper.

HISTORY

In April, 1935, a patent (1) specifying the use of radiant energy of the wave length 10,000 Å. to 16,000 Å. for baking enamel on automobile

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bodies was issued to F. J. Groven and assigned to the Ford Motor Company. Since then incandescent lamps and other equipment furnishing infrared rays have been used on an industrial scale for a variety of heating, baking, and drying operations. Examples of such applications are:

1. Drying paints, varnishes, and lacquers (4, 5, 8, 9, 10, 15, 17, 22).
2. Softening, expanding, or treating metal, cellulose, and plastic materials (6, 10, 11, 12, 13, 18).
3. Baking out radio coils and transformers (7).
4. Drying enamel and glazing solutions (20)
5. Drying in leather, latex, and textile industries (8, 20).
6. Drying chemicals and heating in carrying out chemical reactions (19, 27).
7. Dehydration and preservation of biologicals and food materials (15, 16, 28).
8. Evaporation and removal of water from commercial materials (10, 11, 14, 21, 24, 25).

Drying experiments on tablets made from aspirin, phenacetin and caffeine, phenobarbital tablets, mannitol hexanitrate tablets, and synthetic estrogen tablets have been successful (26).

Radiant heat has also been used for drying purposes in combination with other methods of drying (2, 23).

EXPERIMENTAL

The preliminary work (29) on drying wet tablet granulations by infrared lamps gave results that indicated the usefulness of further study in the same field.

It was then considered necessary to carry out this process of drying on a larger scale to simulate, to some extent, the conditions that prevail in commercial production. Considering the advantages of a conveyorized process over a stationary one in infrared heating operation, a belt conveyor was designed and built so as to secure movement of the granular material under the lamps at a speed which will permit uniform heating with resultant drying to a moisture content conducive to satisfactory compression.

A canvas belt is driven by a motor with desired gearing-down setups at a speed of 1 foot every three minutes. For feeding the granular material so as to have a uniform layer of known thickness, a removable wooden box, with an adjustable metal band to regulate the thickness of the layer of the granular material, was fitted over the feeding end of the belt. The two lamp units as supplied by the Fostoria Pressed Steel Corporation, Fostoria, Ohio, were suspended over the belt with a provision for raising or lowering to increase or decrease the distance between the source of heat and the material being treated. There are two and four lamps, respectively, in each of the lamp units. The capacities of the infrared lamps intended to be used in the process of drying are 250 watt, 375 watt, and 500 watt, respectively.

PART 1

Drying and Compressing of Tablet Granulations.—With a view to determining the optimum conditions with regard to thickness of the bed of granular material (which will be referred to as the thickness hereafter), and distance between the source of heat and the material being treated (which will be referred to as the distance hereafter), the drying operation was carried out on 5-pound batches of sodium bicarbonate granulation. Sodium bicarbonate was selected for this purpose because of its low cost.

Sodium bicarbonate granulation prepared with starch paste as the granulating agent was fed on the belt through the feeding box adjusted to give a definite thickness and dried under 375-watt and 500-watt lamps with the distance kept at 15 inches or 22 inches. The granules carried over the belt under the lamps were collected in a receptacle at the discharge end. The time over which the granules were exposed to infrared heat (which will be referred to as the time hereafter) and the temperature reached at the end of the run (which will be referred to as the temperature hereafter) were noted and recorded. The temperature recorded was the temperature indicated by a thermometer kept for one minute in about 100 Gm. of the heated material collected by scraping across the width of the belt just before it reached the discharge end. The granules collected after the first run had to be subjected to a second equally long or short run under the lamps to dry them to the desired moisture content. Time and temperature were noted and recorded as in the previous run. The dried granulation was properly lubricated and then compressed. No difficulties were encountered during compression. The period of drying, the temperature, the thickness, and the distance were thirty-one minutes, 65°, $^3/_8$ inch, and 15 inches, respectively.

Since lactose and powdered sucrose are very widely used in tablet formulations, lactose and powdered sucrose granulations prepared with starch paste were subjected to the drying operation in the same way as sodium bicarbonate granulation. It was found that lactose and sucrose granulations can be successfully dried by infrared heat in about seventeen to twenty-one minutes, the temperature remaining below 60°. The distance and thickness were 15 inches and $^3/_8$ inch, respectively. These granulations compressed satisfactorily.

The results of drying experiments with sodium bicarbonate, lactose, and sucrose granulations indicate that a distance of 15 inches and a thickness of $^3/_8$ inch could be satisfactory for successful drying, other conditions being more or less identical. A variety of tablet formulas were selected and granulations of the respective formulas were prepared by the use of a suitable granulating agent and were subjected to the drying operation under appropriate infrared lamps for the necessary period of time, keeping the thickness at $^3/_8$ inch and distance at 15 inches in all cases. The temperature of the heated stock was taken as first described

after each run and the time for the entire drying operation was noted in each case. The dried granulation was lubricated properly and compressed. The results of this part of the experimental work are given in Tables I, II, and III.

The legends used in Tables I, II, and III will be interpreted as follows:

T = Talc	B = Binding
ST = Starch	STI = Sticking
MS = Magnesium Stearate	Del. = Substance deliquesces
LC = Less capping	VS = Very soft
P = Picking	SO = Soft

No. 1 = 2% Magnesium Stearate + 2% Starch

PART 2

Moisture Determination.—In order to get an idea of the progress of drying, final moisture content, and the effects of heating on the weight of the materials used in the granulation, moisture determination experiments were carried out in each case in the following manner: A representative sample of the powder mixture to be granulated subsequently was weighed in a previously tared weighing bottle and either subjected to heating in a heating oven at temperatures permissible by the nature of the materials involved for a period ranging from two to five hours to give constant weight, or dried in a vacuum desiccator over sulfuric acid for a period ranging from twenty-four to seventy-two hours to give a constant weight. This procedure was also carried out on a representative sample of the granulated material and a representative sample of the granular material at the end of each run under the lamps.

It was found that moisture of the granulation was cut down approximately by 10%, during the first run, in cases with actual moisture content below 20%. (The time for the first run was seventeen minutes.) In such cases a second run under a few or all the lamps brought the moisture content of the material to a level which permitted successful compression in almost all cases. But in the cases where the actual moisture content was above 20%, heating under the lamps for the same length of time did or did not dry the material so as to be satisfactorily compressed and in some instances a third and even a fourth run was necessary. Examples of such instances with the moisture content after the final run are as follows:

1. Antacid—11.6% after the fourth run.
2. Calcium Carbonate (A)—2.65% after the fourth run.
3. Calcium Carbonate (B)—0.69% after the third run.
4. Buchu and Atropine Compound—7.7% after the third run.

TABLE I.—GRANULATIONS OF INORGANIC COMPOUNDS

Distance: 15 inches
Thickness: 3/8 inch

No.	Name	Granule Size (Mesh)	Bulb (Watts)	Time in Min.	Temp. (Max.), °C.	Results of Drying	Lubricant	Compression
1	Antacid	10	375	63	46.5	Wet	No. 1	P
2	Bismuth Subgallate	10	500	30	63	Dried	No. 1	S
3	Bismuth Subnitrate	16	375	17	48	Dried	No. 1	S
	Bismuth Subnitrate	16	375	24	48	Dried	No. 1	S
4 A	Calcium Carbonate	16	500	50	54	Dried	5% T	B
	Calcium Carbonate	16	500	62	54	Dried	5% T + 5% ST	B
4 B	Calcium Carbonate	10	500	53	60	Dried	No. 1	S
5	Calcium Gluconate	10	500	35	54	Dried	6% T	S
6	Calcium Lactate	10	500	35	58	Dried	6% T	S
7	Calcium Phosphate	16	500	24	59.5	Dried	No. 1	S
8	Calcium Sulfide	10	500	17	61.5	Dried	4% T	S
9	Calomel	10	500	17	61.5	Dried	No. 1	S
10	Ferrous Sulfate	16	500	17	54	Dried	No. 1	S
11	Lithium Citrate	10	500	17	62	Dried	No. 1	S
	Lithium Citrate	10	500	24	62	Dried	No. 1	S
12	Magnesium Salicylate	10	500	21	51.5	Dried	No. 1	S
13	Magnesium Sulfate	10	375	15	...	Dried	2% T	S
14	Magnesium Trisilicate	10	500	35	54	Dried	No. 1	S
15	Mercurous Iodide, Yellow	16	250	24	44.5	Gray on top	6% T	S
16	Potassium Citrate	10	500	17	54	Dried	No. 1	P
17	Sodium Acid Phosphate I	16	375	17	50	Dried	5% T	STI
	Sodium Acid Phosphate II	16	500	14	53	Del.	...	...
	Sodium Acid Phosphate III	16	250	30	45	Dried	5% T + 5% ST	STI
18	Sodium Benzoate	10	500	17	55.5	Dried	No. 1	S
	Sodium Benzoate	10	500	24	55.5	Dried	No. 1	S
19	Sodium Chloride I	10	500	15	51	Dried	No. 1 3% T	C S
	Sodium Chloride II	16	375	17	53	Dried	No. 1	C
20	Sodium Citrate	16	500	17	58	Dried	No. 1	S
21	Sodium Salicylate	10	500	24	59.5	Dried	No. 1	S
22	Strontium Salicylate I	10	500	17	55	Dried	No. 1	C
	Strontium Salicylate II	10	500	14	51	Dried	No. 1 6% T	C LC

TABLE II.—GRANULATIONS OF ORGANIC COMPOUNDS

Distance: 15 inches
Thickness: 3/8 inch

No.	Name	Granule Size (Mesh)	Bulb (Watts)	Time in Min.	Temp. (Max.), °C.	Results of Drying	Lubricant	Compression
1	Aminopyrine I	10	500	30	49	Dried	No. 1	C
	Aminopyrine II	10	500	17	42	Wet	...	...
		..	375	18	51	Dried	No. 1	C
							6% T	LC
2	Caffeine, Citrated	10	500	17	62	Dried	No. 1	S
3	Calcium Pantothenate	10	500	17	51	Dried	5% T	S
4	Charcoal and Pepsin	16	500	20	...	Dried	No. 1	VS
5	Ephedrine Sulfate	10	500	17	62	Dried	1% MS + 1% ST	C
6	Nicotinamide	16	500	21	53	Dried	5% T	S
7	Nicotinic Acid	16	500	17	67	Dried	6% T	S
8	Phenacetin	10	500	24	48.5	Dried	No. 1	S
9	Phenobarbital	10	500	17	53	Dried	No. 1	C
							4% T	S
							4% ST	S
							4% MS	C
10	Riboflavin	16	500	17	55	Dried	5% T	S
11 A	Sulfadiazine	16	500	29	51	Dried	6% T	S
11 B	Sulfadiazine	16	500	29	56	Dried	6% T	S
12	Sulfamerazine	16	500	32	61	Dried	6% T + 3% ST	S
13 A	Sulfanilamide	16	500	17	51	Dried	No. 1	C
13 B	Sulfanilamide I	16	500	17	53	Dried	No. 1	C
	Sulfanilamide II	10	500	17	55.5	Dried	No. 1	C
14	Sulfapyridine	16	500	24	62	Dried	6% T	S
15	Sulfathiazole I	10	375	30	47	Dried	No. 1	S
	Sulfathiazole II	10	500	24	50	Dried	No. 1	S
16	Tannic Acid	10	500	5	...	Fusion	...	..
17	Thiamine Chloride	16	500	17	47	Dried	5% T	S
18	Vitamin C	16	500	24	56	Dried	6% T	S
19	Yeast	16	500	24	55	Dried	No. 1	VS

TABLE III.—COMPOUND GRANULATIONS

Distance: 15 inches
Thickness: 3/8 inch

No.	Name	Granule Size (Mesh)	Bulb (Watts)	Time in Min.	Temp. (Max.), °C.	Results of Drying	Lubricant	Compression
1	Acetanilid, Caffeine and Soda Compound	10	500	30	51	Dried	No. 1	S
2	Acetanilid and Hyoscyamus Compound	10	500	17	51	Dried	5% T	C
3	Aloin, Strychnine, and Belladonna Compound	16	500	24	61	Dried	10% T	S
4	Benzocaine Compound	16	500	33	60 5	Dried	2% MS + 3% ST	S
5	Brown Compound	16				Not Performed		
6	Buchu and Atropine Compound I	16	500	17	...	Damp	..	
	Buchu and Atropine Compound II	16	250	35	...	Dried	7% T	B
7	Calomel, Rhubarb and Colocynth Compound	16	500	17	67	Dried	10%	S
8	Damiana, Nux Vomica, and Zinc Phosphide Compound	16	500	17	61	Dried	10% T	S
9	Extract Cascara Sagrada	16	500	17	63.5	Dried	10% T	S
10	Flatulence	16	500	17	72	Dried	10% T	S
	Flatulence	16	500	24	72	Dried	10% T	S
11A	Laxative I	10	500	17	70	Dried	10% T	S
	Laxative I	10	500	24	70	Dried	10% T	S
11 B	Laxative I	10	500	17	66.5	Dried	5% T	SO
12	Ox Bile, Pepsin, and Pancreatin	16	500	17	...	Dried	No. 1	S
13	Pancreatin and Pepsin	10	500	17	...	Dried	No. 1	S
14 A	Rhubarb and Ipecac Compound	10	500	29	57	Dried	5% T	S
14 B	Rhubarb and Ipecac Compound	16	500	24	57	Dried	5% T	S

PART 3

Assay of the Granulations.—In order to establish absence of any decomposition in the various granulations dried under the infrared lamps, representative samples of granulations were assayed. The nature of the ingredients contained therein suggested probabilities of chemical changes during the drying operation. Such changes, if any, however, could be attributed, in part, to conditions other than heating, to which the materials might have been required to be exposed during the entire process. The results of this part of the experimental are depicted in Table IV.

CONCLUSIONS

From the work done under the present project the following conclusions were drawn:

1. Heating by infrared lamps was rapid and heat distribution was uniform throughout.

2. Infrared heating is applicable to the drying of a variety of tablet granulations provided consideration is given to the nature of the ingredients involved.

3. The following tablet granulations do not lend themselves to successful drying under the infrared heat:

(*a*) Yellow Mercurous Iodide.
(*b*) Sodium Acid Phosphate.
(*c*) Tannic Acid.
(*d*) Dextrose.
(*e*) Buchu and Atropine Compound.

4. The period of drying and the maximum temperature attained during the drying operation depend on the moisture content, distance, thickness, capacity of the bulb, and the nature of the materials involved. (Drying period did not exceed twenty minutes in 50 per cent, thirty minutes in 36 per cent, and forty minutes in 8 per cent of the cases tried. It exceeded forty minutes in 4 per cent of the cases tried. The maximum temperature attained during the drying operation did not exceed 50° in 21 per cent, 60° in 75 per cent, and 70° in 98 per cent of the cases tried. It exceeded 70° in only 1 case.)

5. Granulations with moisture content below 15 per cent can be satisfactorily dried within twenty-five minutes by using lamps of maximum capacity permissible by the nature of the material.

6. No one particular capacity of the bulb will effect satisfactory drying in all cases without

TABLE IV.—ASSAY OF THE GRANULATIONS[a]

No.	Name	Official Requirements	% Labeled	% Found[b]	% of the Labeled Amount
1	Acetophenetidin	≮94% & ≯106% $C_{10}H_{13}O_2N$	92.653	90.252	97.408
2	Aminopyrine	≮93% & ≯107% $C_{13}H_{17}N_3O$	90.5	85.802	94.8
3	Ascorbic Acid	≮95% & ≯120% $C_6H_8O_6$	10.173	10.26	100.86
4	Caffeine, citrated	≮45% & ≯55% of the labeled amount of citrated caffeine	19.55	10.006	51.18
5	Calcium Sulfide U. S. P. IX	Crude Calcium Sulfide contains 55% CaS	19.482	11.41	58.56
6	Ephedrine Sulfate	≮93% & ≯107% $(C_{10}H_{15}NO)_2 \cdot H_2SO_4$	10.345	10.734	103.76
7	Ferrous Sulfate	≮95% & ≯110% $FeSO_4 \cdot 7H_2O$	64.777	61.83	95.45
8	Magnesium Salicylate	.....	93.75	92.013	98.147
9	Mercurous Chloride, Mild	≮92.5% & ≯107.5% HgCl	19.607	21.643	110.38
10	Mercurous Iodide, Yellow	≮91% & ≯109% HgI	5.9535	6.226	104.41
11	Nicotinamide	≮95% & ≯115% $C_6H_6N_2O$	7.0	7.31	104.43
12	Nicotinic Acid	≮95% & ≯115% $C_6H_5O_2N$	9.124	8.64	94.695
13	Phenobarbital	≮94% & ≯106% $C_{12}H_{12}N_2O_3$	9.6385	9.669	100.31
14	Riboflavin	≮95% & ≯120% $C_{17}H_{20}N_4O_6$	0.3704	0.370	100
15 A	Sodium Bicarbonate (starch paste granulated)	≮92.5% & ≯107.5% $NaHCO_3$	95.238	102.238[c]	107.35
15 B	Sodium Bicarbonate (Alcohol granulated)	≮92.5% & ≯107.5% $NaHCO_3$	100	107.03+	107.03
16	Strontium Salicylate	.....	67.67	67.54	99.807
17	Sulfadiazine	≮95% & ≯105% $C_{10}H_{10}N_4O_2S$	58.05	57.594	99.21
18	Sulfamerazine	≮95% & ≯105% $C_{11}H_{12}N_4O_2S$	67.476	65.954	97.744
19	Sulfanilamide	≮95% & ≯105% $C_6H_8N_2O_2S$	64.53	62.93	97.52
20	Sulfapyridine	≮95% & ≯105% $C_{11}H_{11}N_3O_2S$	72.254	71.936	99.56
21	Sulfathiazole	≮95% & ≯105% $C_9H_9N_3O_2S_2$	94.308	95.14	100.9
22	Thiamine Hydrochloride	≮95% & ≯120% $C_{12}H_{17}ClN_4OS$—HCl	0.4576	0.455	99.43

[a] Assay procedures as given in the National Formulary or United States Pharmacopœia, as the case may be.
[b] Figures represent average of four assays. Figures for nicotinamide, nicotinic acid, riboflavin, and thiamine hydrochloride are based on results of assays carried out by the laboratories of Eli Lilly and Company, Indianapolis, Ind.
[c] In the case of sodium bicarbonate, the U. S. P. procedure includes any carbonate that might be present in the granulation. To determine the extent to which the bicarbonate has been changed into carbonate, an assay procedure given in "Scott's Standard Methods of Chemical Analysis" (5) was used. The amount of carbonate in the sodium bicarbonate granulation was found to be 6–13%.

unusually extending the drying cycle or raising temperatures beyond safe levels.

7. Thickness of $^3/_8$ inch and distance of 15 inches seem to enable satisfactory drying. (No attempt was made to determine the optimum thickness and the optimum distance for each case.)

8. No deterioration in the nature of the chemicals that can directly be attributed to the effects of infrared heat could be established.

9. Infrared drying of tablet granulations can be successfully carried out on a large scale by suitably designing a belt conveyor or similar unit to handle the necessary volume of materials.

10. Great savings in floor space are effected by using infrared lamps as a source of heat. The experimental unit, used in this project, having a capacity of 60 pounds, on an average, for an eight-hour working schedule, occupies a floor space of about 80 cubic feet while a unit using any other form of heating to do the same job in ordinary cases could be estimated to require twice as much space.

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Determination of the Stability of Epinephrine by Optical Rotation*

By HAROLD ROSENBLUM, ROBERT GOLDMAN and HERMAN FELDMAN

A method that can be adapted to dilute solutions and oil suspensions of epinephrine for the determination of stability is described. It is recommended that a decrease in optical rotation in liquid preparations of epinephrine preparations should be checked biologically to determine their true physiological activity.

INSTABILITY of epinephrine especially in solution as the hydrochloride has been a matter of interest and concern to all pharmaceutical houses who handle this item. Attempts have been made to overcome this instability with varying degrees of success by the use of stabilizers, usually antioxidants, such as ascorbic acid, amino acetic acid, sodium bisulfite, and also by employing the racemic form as a nonofficial preparation.

The picture is further complicated by the lack of a suitable, rapid, and convenient method for determining the potency of this medicinal. The bioassay is an expensive procedure which requires the services of someone specially trained in the technique of this method and therefore is excluded as a routine method of analysis by the analytical chemist. The colorimetric methods on

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the other hand do not differentiate between the laevo and dextro forms and may show 100% potency when compared with U. S. P. epinephrine, whereas the physiological potency may be slightly higher than 50% if the epinephrine is racemic or has racemized on standing.

In view of the above difficulties this study was undertaken. The method of analysis was based upon the specific rotation of epinephrine when dissolved in water containing a slight excess of hydrochloric acid. Official preparations are epinephrine, epinephrine inhalation (1:100) and epinephrine injection (1:1000). The monograph for the powder fixes the specific rotation in half-normal hydrochloric acid at not less than −50° and not more than −53.5°. No rotation is given for the two solutions.

Upon analyzing the results obtained by determining the specific rotation of about 20 lots of epinephrine powder by dissolving the sample in a slight excess (2–5%) of hydrochloric acid, it was found that in a large majority of samples the rotation was between −51.5° and −52.5°, the average of all lots being −52°. This value was then taken as the theoretical upon which to base the per cent epinephrine in solution. This value furthermore is, for practical purposes, the midpoint in the U. S. P. tolerances.

The difficulties in determining the specific rotation of a 1:1000 solution of epinephrine or a suspension of epinephrine in oil are self-evident.

The observed rotation for a 1:100 solution, using a 200-mm. tube, would be about −1° and for a 1:1000 solution −0.1°. Due to the small reading for the 1:1000 solution, it was found advisable to vacuum concentrate the solution to a 1:100 or 1:200 concentration before determining the rotation. Trial determinations on a 1:1000 solution prepared by diluting a 1:100 solution of predetermined specific rotation with normal saline solution containing 0.1% sodium bisulfite proved successful. The specific rotation of a 1:100 solution was found to be −52°, and after dilution and vacuum concentration −53°. Similar values were found on repeated tests.

For the determination of the epinephrine suspended in oil the procedure had to be modified to remove the epinephrine from the oil before determining the optical rotation. This was accomplished by extracting the epinephrine with 0.1 *N* hydrochloric acid. Values for several samples tested by this method are listed in Table I.

EXPERIMENTAL

The U. S. P. dog assay was used for the biological determination.

TABLE I.—COMPARATIVE VALUES OBTAINED BY BIOLOGICAL ASSAY AND SPECIFIC ROTATION

Sample	Epinephrine by Biological Assay, %	Epinephrine by Specific Rotation,[a] %
Epinephrine injection 1:1000, No. 143	95.7	100.4
Epinephrine injection 1:1000, No. 272	99.4	104
Epinephrine injection 1:1000, No. 045	100	101
Epinephrine in oil 1:500, No. 096	100	97
Epinephrine in oil 1:500, No. 285	100	103

[a] Limit of error ±2.2%.

The method given in "New and Non-Official Remedies 1946," page 297, was used for the colorimetric determination.

Determination of Epinephrine Injection 1:1000 by Optical Rotation.—Place 125 cc. of the epinephrine solution 1:1000, accurately measured, in a 250-cc. standard taper flask and set up the apparatus for vacuum concentration as shown in Fig. 1. The flask contains a capillary tube which dips below the surface of the liquid to be concentrated. Do not use a rubber or cork stopper for the flask as this will cause the solution to become discolored during the concentration.

Concentrate under a vacuum of not more than 15 mm. mercury. Do not allow the water bath to rise above 50°. The vacuum concentration is accomplished as rapidly as possible and the solution concentrated to about 15 cc. Allow the flask to attain atmospheric pressure by opening the capillary tube. Transfer the solution quantitatively to a 25-cc. volumetric flask with the aid of several centimeters of water containing 0.1% sodium bisulfite, adjust the temperature to 25°, and then dilute the solution to 25 cc. with distilled water. Observe the optical rotation in a 200-mm. tube at 25°.

$$\% \text{ of Labeled Strength} = \frac{\alpha \times 10{,}000}{2 \times 52 \times X \times C}$$

α = observed rotation
X = labeled strength in per cent
C = the number of times the solution has been concentrated

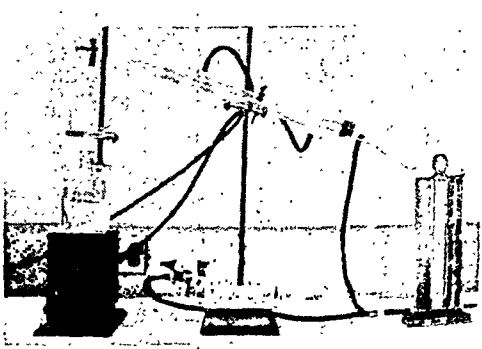

Fig. 1.—Apparatus for vacuum concentration of epinephrine solutions.

Determination of Epinephrine in Oil by Optical Rotation.—Shake the ampuls or vials containing the preparation to disperse thoroughly the suspended epinephrine, and then remove the material with the aid of a syringe and needle. Collect the material in a 125-cc. glass stoppered flask, shake well, and withdraw an aliquot sufficient to furnish 100 mg. of epinephrine and transfer to a dry separatory funnel. Add exactly 20 cc. of 0.1 *N* hydrochloric acid and shake the mixture well for five minutes. Allow to stand until the layers separate, draw off the lower aqueous layer and filter through a dry filter into a dry container. Transfer the filtrate to a 200-mm. polariscope tube and determine the angular rotation of the solution at 25°.

The calculations are the same as for the 1:1000 aqueous solution.

DISCUSSION

Solutions of epinephrine hydrochloride (1:100) of several manufacturers were assayed biologically, colorimetrically and by their specific rotation. The results are recorded in Table II. With deteriorated solutions the colorimetric method gave the highest results, the biological method the next highest, the optical rotation the lowest results. This can be attributed to racemization of the *l* form to the *d* form. For example, Lot No. 092, when freshly prepared, showed approximately 100% epinephrine by all three methods. Upon standing several months, the solution apparently partially racemized as indicated by the fact that the colorimetric method still showed 100% of potency, the bioassay only 91% and the optical rotation 70%.

This lot shows by optical rotation that 15% of the laevo form still predominates. The colorimetric method still shows 93% epinephrine. It appears therefore that 39% has been converted to the dextro form, yielding 78% of racemic epinephrine. Therefore, 39%, which is the laevo fraction of the racemic epinephrine plus 15% laevo equals 54%. The biological method should show about 54% activity. Actually 55% was found which can be considered within the experimental error.

A comparison of the biological assay with the average of the physical measurements for each sample (see Table II) shows a fairly close agreement in several instances. The accuracy of each determination is subject to an error of approximately ±3%. This error may be cumulative, and the actual variation between biological and physical measurements can be about ±10%.

However, these results indicate that by averaging the values obtained by the colorimetric method and the specific rotation (Table II), the biological activity can be estimated. The concentration of an unknown solution by optical rotation using a 200-mm. tube can be calculated from the formula:

$$\% \text{ Epinephrine} = \frac{\alpha \times 100}{2 \times C \times 52}$$

C = number of times solution has been concentrated. For example: 100 cc. concentrated to 20 cc. = 5 times.

This value averaged with the colorimetric determination should approximate the biological activity.

TABLE II.—EPINEPHRINE DETERMINATION

Sample	Epinephrine By: Colorimetric Assay, %	Specific Rotation, %	Biological Assay, %	Average of Specific Rotation and Colorimetric Assay, %
Epinephrine, inhalation 1:100, No. 190, prepared on 7-3-47	115	105	97	110
Epinephrine, inhalation 1:100, No. 190, retested on 4-29-48	115	70	100	92.5
Epinephrine, inhalation 1:100, No. 092, prepared on 4-1-47	102	100	96.4	101
Epinephrine, inhalation 1:100, No. 092, retested on 4-29-48	100	70	91	85
Epinephrine, inhalation 1:100, C2029	102.5	15	55	59
Epinephrine, inhalation 1:100, 15928	93	15	55	54

The difference in the results can be postulated as follows: upon racemizing, one molecule of epinephrine is converted to the dextro isomer which neutralizes the rotation of another laevo molecule. Therefore, if half the epinephrine racemized to the dextro form, there would be no rotation and the solution would have slightly more than 50% of its potency (due to the slight activity of the *d* form) by biological assay. The solution would still show 100% of potency by colorimetric assay due to the fact that this method cannot distinguish between the dextro and laevo forms. The results of Lot No. 15928 as shown in Table II will best illustrate this point.

The above results show that racemization or deterioration is readily detected by checking the optical rotation periodically. Furthermore, it is our experience that if racemization occurs in solution, the most rapid fall in rotation will occur within a month after the solution is prepared. The rotation drops to about −35°, after which further racemization does not occur at all or will progress at a very much slower rate. The cause for the racemization has not been definitely placed and is under further investigation.

The widespread belief that the loss in physiological potency invariably is indicated by the production of a brown color and precipitate is

erroneous. The addition of bisulfite does not prevent racemization or decomposition but may prevent discoloration from decomposition products. A solution of epinephrine hydrochloride containing bisulfite may lose a large part of its physiological properties and yet it may remain colorless or turn only slightly yellowish. The limitation on this method for determining the potency by optical rotation of the solution is of course the brown color which may be produced by oxidation in some solutions. However, this color in itself would automatically eliminate the material from sale or from being used by the patient. For the purpose of controlling the product and determining the shelf life, the optical rotation method has been found to be a simple, rapid, and inexpensive procedure.

SUMMARY

1. Solutions of epinephrine hydrochloride can be constantly checked for stability by determining the optical rotation.

2. Deterioration can be detected most readily by this means since the effect is most pronounced upon the rotation.

3. Evidence is presented that deterioration can be caused by racemization.

4. The method can be adapted to solutions of 1:1000 and also to oil suspensions.

5. A solution showing a drop in optical rotation should be checked biologically to determine its true physiological potency.

The Bactericidal Action of Oxygen-Liberating Substances on Oral Microorganisms. II. Studies with Oral Spirochetes*

By WILLIAM A. NOLTE,† MARGUERITE A. EDWARDS‡ and THOMAS C. GRUBB

Two strains of oral spirochetes, isolated from cases of Vincent's infection, were cultured in a thioglycollate-blood-infusion medium and inoculated into various concentrations of sodium perborate, hydrogen peroxide, and a neutralized perborate dentifrice. Subcultures made after thirty seconds, one and two minutes' contact were incubated at 37° and examined by the dark-field method after seven and fourteen days' incubation. Controls included in each experiment warranted the conclusion that the absence of growth in the subculture tubes resulted from the germicidal action of the oxygen-liberating substances *per se*. The perborate dentifrice diluted 1:10 with water, saliva or saliva plus 10% serum killed billions of spirochetes in less than thirty seconds, in most instances. The perborate dentifrice, hydrogen peroxide and sodium perborate diluted with water exhibited approximately equal spirochetacidal action when compared on the basis of equal oxygen content. Although all three products were equally effective in the lower dilutions usually employed for oral use, the strong alkalinity of sodium perborate and the high acidity of commercial hydrogen peroxide solutions are considered undesirable properties of products intended for oral application.

RECENTLY a paper (1) from this laboratory appeared which described the rapid germicidal action of sodium perborate, hydrogen peroxide and a neutralized sodium perborate dentifrice on fusiform bacilli suspended in water, saliva, or saliva plus serum. In order to complete the study of the action of oxygen-liberating substances on oral microorganisms, especially those associated with Vincent's infection, experiments with these substances were carried out using two strains of oral spirochetes as the test organisms.

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EXPERIMENTAL

Two strains of oral spirochetes (Nos. 69 and 78) isolated from cases of Vincent's infection[1] were cultivated in the following medium:

Sodium thioglycollate, Gm.	1
Dextrose, Gm.	10
Agar, Gm.	0.5
Beef infusion with 1.0% thiopeptone, cc.	1000

The medium was adjusted to *p*H 7.8, tubed in 10 cc. amounts and autoclaved twenty minutes at 15 lb. Just before use, 0.5 cc. of citrated rabbit's blood was added to each tube. The stock cultures were continuously maintained by inoculating 0.5

[1] The writers are indebted to Dr. Ruth Wichelhausen for the cultures used in this study.

cc. of a ten-day-old culture into fresh tubes which were in turn incubated ten days at 37° before transferring to fresh media.

Since it was believed important to know the number of organisms employed in the germicidal tests, a method was devised, similar to the Breed count in principle, which was used to count the number of spirochetes per dark field and translated into the number of organisms per cubic centimeter. When checked by growth of the organisms in serial dilutions, the dark field counts showed close agreement

In the first series of experiments the germicidal action of a neutralized sodium perborate dentifrice[2] was determined as follows:

Five-tenths of a cubic centimeter of a ten- to twelve-day-old culture of spirochetes was added to 5 cc. of the neutralized perborate dentifrice diluted 1:5 in distilled water, undiluted sterile saliva or undiluted sterile saliva plus 10% rabbit serum. After a contact interval of thirty seconds, one and two minutes at room temperature, a 4-mm. loopful of the mixture was transferred to tubes of the spirochete medium which were incubated at 37° and examined for growth by the dark field method after seven and fourteen days' incubation.

Two controls were always included in each experiment reported in this paper. The first was a "carry-over" control which consisted of inoculating a loopful of the most concentrated dilution of the dentifrice, sodium perborate or hydrogen peroxide used in each test, into the subculture medium, followed by a loopful of the test organisms. Since these controls always showed growth it indicated that the amount of oxygen-liberating substance carried over by the loop did not produce bacteriostasis in the subculture medium and hence any tubes failing to show growth had been inoculated with organisms killed by the test substances. The second type of control included in all experiments consisted of the inoculation of 0.5 cc. of the test organism into water, undiluted saliva or undiluted saliva plus 10% serum and allowed to remain in contact with these substances for the longest period of medication contact employed in each experiment (i.e., two minutes), before a loopful was transferred to the subculture medium. Since these "menstruum" controls always showed growth in the subculture tubes it was concluded that contact of the spirochetes with water, saliva or saliva plus serum *per se* did not contribute significantly to the killing of the test organisms. Since these two types of controls showed satisfactory results in each of the experiments reported, it is believed that the failure of growth to appear after contact with the various oxygen-liberating agents represents the results of a true germicidal test.

In the first series of experiments a 1:5 dilution of the dentifrice was employed for testing because it has been estimated that this is the approximate concentration of a powdered dentifrice in the mouth under the usual conditions of use. The results of this experiment indicated in Table I show that both spirochete strains were killed after not more than thirty seconds' contact with the neutralized perborate dentifrice diluted with water, saliva, or saliva plus serum. Since the inoculum in these experiments contained approximately 18,580,000,000 spirochetes (the majority of which appeared to be motile), it is evident that the dentifrice was markedly germicidal for these organisms.

Since the perborate dentifrice proved so effective in killing spirochetes in low dilutions, a study was made to determine its activity in higher dilutions. The methods employed were the same as those described for the preceding experiments except that dilutions of the dentifrice from 1:10 to 1:80 were made in sterile water, saliva, or saliva plus 10% serum. The inocula contained approximately 20,000,000,000 spirochetes. The results of this test are shown in Table II.

TABLE I.—SPIROCHETACIDAL ACTION OF THE NEUTRALIZED PERBORATE DENTRIFRICE DILUTED 1:5 ON ORAL SPIROCHETES

Contact Time	Strain No. 69			Strain No. 78		
	Water Dil.	Saliva Dil.	Saliva + Serum Dil.	Water Dil.	Saliva Dil.	Saliva + Serum Dil.
30 sec.	—[a]	—	—	—	—	—
1 min.	—	—	—	—	—	—
2 min.	—	—	—	—	—	—
Control	+[b]	+	+	+	+	+

[a] — = No growth in subculture tubes.
[b] + = Growth in subculture tubes.

The action of the dentifrice on spirochetes shows the usual time-dilution relationship found with bacteria subjected to germicides; i.e., the higher the dilution the greater the length of time required to kill the organisms. In the case of strain No. 69 the presence of serum in the saliva caused some reduction in the activity of the dentifrice. Strain No. 78 showed some inconsistencies which are difficult to interpret, since the presence of saliva alone had a greater inhibitory action on the dentifrice than saliva plus serum. In general the dentifrice killed the spirochetes within one minute when diluted 1:20.

Since sodium perborate or hydrogen peroxide is frequently used in dental practice to treat oral infections it was considered of interest to compare their *in vitro* bactericidal action on oral spirochetes with that of the neutralized perborate dentifrice. The test methods were the same as those used in

[2] The dentifrice (Neutrox) has the following composition: sodium perborate monohydrate (U. S. P. dehydrated), 30%; monocalcium phosphate, 30%; tricalcium phosphate, 23.87%; [illegible] ate, 10%; saccharin soluble, 0.3%; [illegible] menthol 0.33%; oil of peppermint, 0.[illegible] 0.65%; oil of anise, 0.042%; liquid [illegible] 1.81%; and sodium lauryl sulfoacetate, [illegible] (U. S. Patent No. 2,094,671.)

TABLE II.—SPIROCHETACIDAL ACTION OF THE NEUTRALIZED PERBORATE DENTIFRICE DILUTED 1:10 TO 1:80 ON ORAL SPIROCHETES

Contact Time	Strain No. 69 1:10	1:20	1:40	1:80	Strain No. 78 1:10	1:20	1:40	1:80
			Water Dilutions					
30 sec.	−[a]	+[b]	+	+	−	−	−	+
1 min.	−	−	+	+	−	−	−	+
2 min.	−	−	+	+	−	−	−	−
Control	+				+			
			Saliva Dilutions					
30 sec.	−	+	+	+	+	+	+	+
1 min.	−	−	+	+	−	+	+	+
2 min.	−	−	+	+	−	−	+	+
Control	+				+			
			Saliva plus Serum Dilutions					
30 sec.	+	+	+	+	−	−	+	+
1 min.	−	+	+	+	−	−	−	+
2 min.	−	+	+	+	−	−	−	−
Control	+				+			

[a] − = No growth in subculture tubes.
[b] + = Growth in subculture tubes.

the preceding experiments. In preparing the dilutions of the substances to be tested, the concentration of available oxygen per Gm. or cc. was first determined and then dilutions in sterile water were made so that the corresponding dilution of each substance contained the same amount of available oxygen; e.g., a 1:50 dilution of sodium perborate contained the same amount of available oxygen as a 1:20 dilution of the perborate dentifrice. In these experiments the strain No. 69 inoculum contained approximately 19,509,000,000 spirochetes and strain No. 78 contained 25,083,000,000 spirochetes. (See Table III.)

The comparison of the three oxygen-liberating substances on oral spirochetes shown in Table III indicates that most have approximately the same spirochetacidal action. This suggests that the principal agent in each substance is the hydrogen peroxide or nascent oxygen liberated in solution.

DISCUSSION

The results of this study indicate that oxygen-liberating substances have a rapid spirochetacidal action *in vitro;* even in the presence of saliva and serum included to simulate *in vivo* conditions of use. A direct comparison of the relative effectiveness of these substances on fusiform bacilli previously reported (1) and on oral spirochetes is not possible because inocula containing approximately a hundred times greater number of organisms were used in the spirochete experiments. However, with this factor taken into consideration the results suggest that the oxygenating substances tested kill spirochetes more rapidly than they do fusiform bacilli. Since it has been demonstrated in the two papers of this series that oxygen-liberating substances are actively germicidal *in vitro* against both types of organisms associated with Vincent's

TABLE III.—COMPARISON OF SPIROCHETACIDAL ACTION OF THE NEUTRALIZED PERBORATE DENTIFRICE WITH SODIUM PERBORATE AND HYDROGEN PEROXIDE

Contact Time	Strain No. 69 1:10	1:20	1:40	1:80	1:160	Strain No. 78 1:10	1:20	1:40	1:80	1:160
				Perborate Dentifrice						
30 sec.	−[a]	+[b]	+	+	+	−	−	+	+	+
1 min.	−	−	+	+	+	−	−	+	+	+
2 min.	−	−	−	−	+	−	−	+	+	+
Control	+					+				
				Hydrogen Peroxide[c]						
	1:4	1:8	1:16	1:32		1:4	1:8	1:16	1:32	
30 sec.	+	+	+	+		+	+	+	+	
1 min.	−	−	+	+		−	+	+	+	
2 min.	−	−	−	−		−	−	−	−	
Control	+					+				
				Sodium Perborate[d]						
		1:50	1:100	1:200	1:400		1:50	1:100	1:200	1:400
30 sec.		+	+	+	+		+	+	+	+
1 min.		−	+	+	+		+	+	+	+
2 min.		−	−	−	−		−	−	+	+
Control		+					+			

[a] − = No growth in subculture tubes.
[b] + = Growth in subculture tubes.
[c] Hydrogen peroxide, U. S. P.
[d] Sodium perborate monohydrate, U. S. P.

infection, it is believed that these experiments justify the widespread reliance placed in these agents for the treatment of this infection.

The choice between the three oxygenating substances for oral usage, briefly discussed in the previous report (1), may be considered here. While sodium perborate, hydrogen peroxide and the neutralized perborate dentifrice all appear to be equally effective against oral spirochetes and fusiform bacilli in the low dilutions commonly employed in the mouth, the fact that sodium perborate is strongly alkaline (*p*H 9.9) and commercial hydrogen peroxide is quite acidic (*p*H 3.7–4.9), suggests that these two agents are not so desirable for oral application as one which has been carefully buffered to an approximately neutral state (2) like the neutralized perborate dentifrice employed in this study.

SUMMARY

Carefully controlled *in vitro* tests with sodium perborate, hydrogen peroxide and a neutralized perborate dentifrice indicated that these substances were rapidly spirochetacidal for two strains of oral spirochetes. The perborate dentifrice diluted 1:10 with water, saliva, or saliva plus 10 per cent serum killed approximately 20,000,000,000 spirochetes in less than one minute. Although the oxygen-liberating substances included in this study appear to have approximately equal spirochetacidal activity when compared in dilutions of equal oxygen content, the perborate dentifrice would appear to be the most suitable for oral application because of its neutral reaction.

REFERENCES

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(2) Manchey, L. L., and Lee, S., THIS JOURNAL, 26, 890(1937).

The Determination of Thymol and Carvacrol in Spanish Thyme Oils*

By EDWARD E. LANGENAU

The definition of thyme oil in The National Formulary, Eighth Edition, excludes those oils which contain carvacrol as the main phenolic constituent. However, in the monograph no analytical procedure is incorporated to distinguish between the carvacrol type and the thymol types of oils. This paper was written in order to initiate the accumulation of data which will lead to a satisfactory test. A procedure is given for the separation of phenols from thyme oils; the behavior of known mixtures of such separated phenols at fixed temperatures, when seeded with a crystal of thymol, is described.

ACCORDING to Guenther (1, 2), the commercial production of thyme oils in Spain results in two main types of oils:

1. Red thyme oils distilled from *Thymus vulgaris*, L. and *Thymus zygis*, L.; the phenolic constituents of this type of oil consist mainly of thymol, and contain only a small percentage of carvacrol. This oil is distilled in the provinces of Murcia and Almeria, an area entirely separated from the producing sections of Spanish origanum oil.

2. Origanum oils distilled from *Thymus capitatus*, Lk., now considered a separate genus, *Coridothymus capitatus* Rchb.; the phenolic constituents of this type consist mainly of carvacrol, and contain only a small percentage of thymol. This plant grows in the southern part of Spain (Andalusia), particularly in the provinces of Malaga, Cadiz, Huleva, Sevilla, and on the borders of Cordoba.

Oils containing thymol as the principal phenolic constituent are preferred generally in the trade to oils containing carvacrol. This preference may well be due to the fact that thyme oils were frequently used to replace the Indian ajowan seed oils as a source for crystalline thymol. In the United States, the manufacture of synthetic thymol on a large scale has resulted in a product that meets all U. S. P. requirements at a lower cost than natural thymol isolated from thyme oils.

For use in flavoring, the thyme oils are usually preferred because of their somewhat finer flavor character. An important quantitative difference in the phenol contents exists between the two types: Thyme oils usually contain about 50% phenols; origanum oils, about 65 per cent to 70 per cent phenols.

In the course of the following experiments, thyme oils were reconstituted from the separated

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nonphenolic portions of Spanish oils and from phenolic mixtures of thymol and purified carvacrol. In odor, little difference could be detected as the thymol-carvacrol ratio was altered. In view of these findings, both oils should be entirely satisfactory for imparting a pleasant phenolic odor to soaps; this is the most important commercial use of thyme and origanum oils today. For this purpose, the origanum oils offer the advantages of lower cost and greater strength.

For use in pharmaceuticals and medicinals, there is still some question as to the relative merits of the two types of oils. Much conflicting literature exists. It appears, however, that the medicinal value of the oils depends upon the presence of the phenols, thymol and carvacrol, and that both phenols have medicinal value. Livingston (3) made a careful comparison of thymol and carvacrol and found that toxicity and relative anthelmintic value of the two phenols were practically identical. Martindale (4) concluded that carvacrol is almost as actively germicidal as its isomer, thymol.

The National Formulary, Eighth Edition (5), defines thyme-oil as the "volatile oil distilled from the flowering plant of *Thymus vulgaris* Linné or *Thymus zygis* Linné and its variety *gracilis* Bois (Fam. *Labiatae*)." This definition excludes the carvacrol type of oil.

The following experiments were undertaken in an attempt to develop a practical laboratory method which would determine the percentage of thymol in the phenolic portion of the oil, and thus distinguish between the thymol and carvacrol types of oils.

Mayor (6) has reviewed several methods for the determination of total phenols in thyme oils and has described differences in the behavior of carvacrol and thymol. In the review, Mayor has described briefly several methods for the estimation of thymol in the presence of carvacrol; most of these are of value for the commercial separation of the two phenols, but leave much to be desired for a routine analytical procedure. The method of Sage and Dalton (7) appears the most promising; these authors determined congealing points for thymol-carvacrol mixtures and for mixtures of these phenols recovered from thyme oils of known composition. Their findings, however, are at variance with the data set forth in this paper.

EXPERIMENTAL

Part A—Phenols

Thymol.—The thymol used in the following experiments was of U. S. P. quality. The melting point was 49.5°–50.5° when determined in a capillary tube according to the procedure outlined in the U. S. P. XIII for testing materials of Class I (8). It showed a congealing point of +49.4° when supercooled to +40° and tested as described below under "Procedure." The thymol was considered to be of sufficient purity for use without further purification.

Carvacrol.—A commercial grade of carvacrol, warranted free from thymol by the manufacturer, was fractionated *in vacuo* with an 8-inch Vigreau column. The fraction +119°–121° at 15 mm. was collected; this represented 75% of the original product. The analysis of the fraction follows: $d_{25°}^{25°}$ = 0.977; $\alpha_D^{25°}$ = ±0°; $n_D^{20°}$ = 1.5236; solubility 20°—clearly soluble in 2 volumes and more of 70% alcohol; solubility in alkali—clearly soluble in 10 volumes of KOH T.S.; upon standing, the solution developed a faint opalescence.

These data are in good agreement with the values reported in the literature for pure carvacrol (9).

To assure the absence of thymol, the following tests were carried out:

1. The carvacrol was supercooled to −20° and seeded with a small crystal of thymol: no growth of thymol crystals was observed after four hours at this temperature.

2. A mixture of 9.500 Gm. of thymol and 0.500 Gm. of carvacrol showed a congealing point of +46.2° when supercooled to +40° and tested as described below under "Procedure"; a second mixture of 9.500 Gm. of thymol and 0.500 Gm. of eugenol of U. S. P. quality showed the identical congealing point, +46.2°, when treated in a similar manner. If the carvacrol had contained thymol, a difference in congealing points would have been expected.

3. The carvacrol was tested for the presence of thymol by the procedure of Klingstedt and Sundström (10). The phenol was converted to the methyl ether by means of dimethyl sulfate. This ether was saturated with gaseous hydrogen chloride and a solution of sodium nitrite was added; no crystalline nitroso compound was formed. According to Klingstedt and Sundström, if thymol is present, a crystalline nitroso thymol will result.

Mixtures of Thymol and Carvacrol.—Mixtures of thymol and carvacrol were prepared from the phenols described above; the percentage of thymol was varied from 0% to 100%, by weight, in 10% steps. The analyses of the eleven samples thus prepared are given in Table I.

TABLE I.—ANALYSES OF MIXTURES OF PHENOLS

No	Composition, % Thymol	Carvacrol	$d_{25°}^{25°}$	$n_D^{20°}$
0	0	100	0.977	1.5236
1	10	90	0.977	1.5235
2	20	80	0.976	1.5233
3	30	70	0.976	1.5232
4	40	60	0.975	1.5231
5	50	50	0.975	1.5230
6[a]	60	40	0.975	1.5230
7[a]	70	30	0:974	[b]
8[a]	80	20	[b]	[b]
9[a]	90	10	[b]	[b]
10[a]	100	0	[b]	[b]

[a] At a temperature of 25°, No 6 through No. 10 showed a spontaneous separation of crystals within twenty-four hours. (No. 0 through No. 5 showed no such crystallization.)
[b] Mixture crystallized spontaneously during determination.

The differences in specific gravity and refractive index were insufficient for establishing the percentages of the phenols in the mixtures. The behavior of the mixtures at various temperatures was therefore investigated.

The use of the true congealing point did not prove entirely satisfactory. The congealing point was found to be dependent upon the degree of supercooling; with high percentages of thymol the degree of supercooling had little effect, but as the percentage of thymol was decreased, the effect of supercooling became very noticeable. Furthermore, at low temperatures and high percentages of carvacrol, the mixtures became very viscid and the crystals of thymol formed so slowly that no appreciable rise in temperature was observed in some cases.

The use of the end point of the melting range (the point at which the last trace of crystalline material disappears) also proved unsatisfactory. Overheating of the mixtures occurred frequently because of the high viscosity; temperature equilibrium was attained very slowly. Furthermore, the size of the crystals present, which is dependent upon the speed of crystallization, also affected the titer; large crystals dissolved slowly, so that the temperature of the mixture could be raised many degrees above the true melting point.

If the mixtures were maintained at a fixed temperature and a crystal of thymol added as a seed, the mixture would then crystallize if it were in a supercooled state. Since the mixtures could readily be supercooled many degrees below the true melting point, the introduction of the seed of thymol was essential to obtain reproducible results. Upon this behavior were the mixtures of phenols classified.

The procedure described below was used to study the behavior of the phenolic mixtures at various fixed temperatures. (See Table II.)

Procedure.—Into a test tube, 20 mm. in diameter and 100 mm. in length, introduce about 10 Gm. of the phenols. Insert a thermometer graduated in 0.1°. Warm gently until the contents are completely melted and a homogeneous liquid is obtained, free from any crystalline material. Insert the tube through a bored cork into a larger test tube, 40 mm. in diameter and 200 mm. in length, which is to act as an air jacket. Immerse the larger test tube to a depth of 170 mm. in a water bath maintained at a fixed temperature. Allow the contents of the inner tube to cool slowly without agitation until the temperature of the water bath is attained. Then introduce a small crystal of thymol to act as a seed and stir the contents vigorously with the thermometer, rubbing the latter on the sides of the tube with an up-and-down motion in order to induce rapid crystallization. Continue the stirring and rubbing as long as the temperature rises; record the highest point as the congealing point. If crystallization does not occur immediately, continue the stirring for five minutes and observe the condition of phenols to ascertain whether or not there is any haze or turbidity, resulting from the presence of small crystals throughout the liquid.

Part B—Thyme Oils

To study the behavior of thyme oils containing known percentages of thymol and carvacrol, such oils were prepared from the nonphenolic portion of a Spanish thyme oil and the thymol and carvacrol described in Part A.

Nonphenolic Portion of Thyme Oil.—A Spanish thyme oil of direct importation was used. The oil met all N. F. VIII requirements and had the following properties: $d^{25°}_{25°} = 0.9245$; $\alpha^{25°}_{D} =$

TABLE II.—BEHAVIOR OF MIXTURES OF PHENOLS

No.	Composition, % Thymol	Carvacrol	Mixture Cooled to 0°	5°	10°	15°	20°	25°	30°	35°	40°	45°	50°
0	0	100	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
1	10	90	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
2	20	80	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
3	30	70	C.S. Notes 1 & 2	C.S. Notes 1 & 3	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
4	40	60	C.S. Note 4	C.I. Note 4	C.S. Note 1	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
5	50	50	Note 5	C.I. C.P. = 15.5°	C.I. C.P. = 18.4°	C.I. C.P. = 20.5°	C.S. Note 1	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
6	60	40	Note 5	C.I. C.P. = 17.0°	C.I. C.P. = 20.0°	C.I. C.P. = 21.6°	C.I. C.P. = 24.1°	C.S Note 1	N.C.	N.C	N.C.	N.C.	N.C.
7	70	30	Note 5	Note 5	Note 5	Note 5	C.I. C.P. = 29.0°	C.I. C.P. = 31.4°	C.I. C.P. = 33.1°	C.S. Note 1	N C.	N.C.	N.C.
8	80	20	Note 5	Note 5	Note 5	Note 5	C.I. C.P. = 33.5°	C.I. C.P. = 35.4°	C.I. C.P. = 37.4°	C.I. C.P. = 39.0°	C.S. Note 1	N.C.	N.C
9	90	10	Note 5	Note 5	Note 5	Note 5	C.I. C.P. = 40.0°	C.I. C.P. = 41.5°	C.I. C.P. = 42.3°	C.I. C.P. = 43.4°	C.I. C.P. = 43.4°	N.C.	N.C.
10	100	0	Note 5	Note 5	Note 5	Note 5	Note 5	Note 5	C.I. C.P. = 49.0°	C.I. C.P. = 49.2°	C.I. C.P. = 49.4°	C.I. C.P. = 49.4°	N.C.

Key: N.C. = no crystallization observed; C.I. = crystallizes immediately; C.S. = crystallizes slowly; C.P. = congealing point.

NOTE 1—No appreciable temperature rise observed.
NOTE 2—Mixture becomes turbid owing to presence of thymol crystals.
NOTE 3—Mixture becomes hazy owing to presence of thymol crystals.
NOTE 4—Temperature rise is slow; congealing point is not sharp and is not easily reproducible.
NOTE 5—Attempts to supercool to this temperature were unsuccessful; mixtures crystallized spontaneously.

$-1°56'$; solubility 25°—clearly soluble in 1 to 1.5 volumes of 80% alcohol and more; $n_D^{20°} = 1.4989$; phenol content = 48.5%; color—red.

Two and a half liters of the oil were stirred and shaken thoroughly with 12.5 L. of potassium hydroxide T.S. The mixture was permitted to separate overnight and the oily layer (1325 cc.) siphoned off. After two additional washings with 5 volumes (6,625 cc.) of potassium hydroxide T.S., the separated oily layer was shaken with 200 cc. of water and then with two 200-cc. portions of a saturated sodium chloride solution. The separated oil was dried with anhydrous sodium sulfate and filtered. The analysis of this nonphenolic portion of thyme oil is: $d_{25°}^{25°} = 0.878$; $\alpha_D^{25°} = -2°44'$; $n_D^{20°} = 1.4792$; solubility 25°—clearly soluble in 0 to 0.5 volumes 90% alcohol and more, hazy in 10 volumes of 80% alcohol; apparent phenol content = 1.0%.

The apparent phenol content probably is due to the solubility in water of some constituent other than a phenol, since upon separation of the aqueous potassium hydroxide layer (obtained from the assay) and subsequent acidification, no oily separation was observed. Extraction of the acidified aqueous layer with ether, and subsequent drying and removal of the ether gave only a trace of an oily liquid with a camphoraceous odor. This oily liquid did not have the characteristic phenolic odor of carvacrol or thymol; it gave no color reaction with a solution of ferric chloride (1:1000).

Reconstituted Thyme Oils.—Thyme oils were reconstituted by combining 50.0 Gm. of this nonphenolic portion and 50.0 Gm. of the mixtures of pure phenols containing 0% to 100% thymol. The analyses of the eleven samples of reconstituted oils is given in Table III.

Separation of Phenols.—The phenols were separated from these reconstituted thyme oils by the following method: Introduce 25 cc. of the oil into a 500-cc. separatory funnel. Add 250 cc. of potassium hydroxide T.S. Shake the mixture thoroughly for five minutes and then permit the two layers to separate overnight. Draw off the lower aqueous layer and filter into a 1000-cc. separatory funnel through a fluted filter paper previously wetted with water.[1] Add 100 cc. of dilute sulfuric acid (1:3) and 100 cc. ether. Shake thoroughly and permit the two layers to separate; draw off the lower aqueous layer into a second 1000-cc. separatory funnel; retain the upper ether layer. Extract the aqueous layer a second time with 100 cc. of ether; the aqueous layer should now be colorless. Combine the two ether layers, dry with 25 Gm. of anhydrous sodium sulfate and filter; wash out any oil adhering to the sodium sulfate with a 25-cc. portion of ether and add this washing to the dried ether solution. Evaporate the ether in a Pyrex evaporating dish which has a top diameter of 90 mm. and a height of 50 mm. The evaporating dish should be heated on a steam bath in such a manner that only the flattened bottom is in contact with the steam; a few small clay chips should be added to prevent bumping and to hasten evaporation. Add initially about 75 cc. of the ethereal solution to the dish, and when this volume is reduced to about 50 cc., add a further 25-cc. portion of the ethereal solution. After all of the solution has been added in this manner, continue the evaporation until the thin small stream of ether bubbles rising from the clay chips just ceases. Continue the heating on the steam bath for an additional thirty minutes; remove the evaporating dish and pour the contents into a test tube. Determine the behavior of the separated phenols at various fixed temperatures using the technique described under the paragraph "Procedure" in Part A.

The phenols separated from the eleven samples behaved as indicated in Table IV.

It should be noted that the phenolic mixtures recovered from the reconstituted thyme oils (Table IV) behave somewhat differently from the original mixtures (Table II). This variation in behavior is due to many contributing factors, some of which are discussed below.

1. The complete removal of the ether from the phenols is essential as evidenced by the following experiment. About 5 Gm. of thymol (congealing point = +49.4°, when supercooled to +40° and seeded) was dissolved in 100 cc. of ether, and the ether removed by the technique described in the procedure for the separation and recovery of the phenols from thyme oil. When the stream of ether bubbles just ceased, the evaporating dish was removed from the steam bath and permitted to cool; no odor of ether was observed. When cooled to 40° and seeded, no crystallization occurred; when cooled to +30° and seeded, crystallization occurred with a rise of temperature to only 35.5°. The evaporating dish was then permitted to stand open overnight to permit the residual ether to evaporate; when seeded at a temperature of +35°, a congealing point of 41.8° was obtained. Additional heating for 10 minutes raised the congealing point to +48.0° when seeded at +40°. The experiment was repeated, but the evaporating dish was heated for a period of thirty minutes after the stream of ether bubbles just ceased; a congealing point of +49.2° was obtained when seeded at +40°. Hence this thirty-minute heating period was incorporated in the procedure.

2. The heating period of thirty minutes alters the composition of the phenolic mixture because of a difference in the vapor pressure of thymol and carvacrol. This factor affects somewhat the accuracy of the experimental results.

About 10 Gm. of a mixture of 70% thymol–30% carvacrol was heated in a glass evaporating dish as described in the procedure. The loss of weight and the congealing point were determined after thirty minutes and after sixty minutes. The following data were obtained.

	Original	After 30 Min.	After 60 Min.
Total loss	....	3.3%	6.4%
Congealing point, supercooled to +30° and seeded	+33.1°	+32.3°	+31.3°

3. The alkaline solution used for the separation of the phenols has a definite adverse effect upon thymol.

[1] The solution is not washed with ether at this point; this is to prevent loss of the phenols, since both carvacrol and thymol can readily be washed out of an alkaline solution of their salts with ether. This fact has been reported in the literature (11) and was confirmed by the author.

Upon dissolving 12.5 Gm. of thymol in 250 cc. of potassium hydroxide T.S. a clear colorless solution resulted. Upon standing overnight, this solution developed a yellow color but remained clear. Upon acidification and subsequent recovery of the thymol, 12.2 Gm. of a deep yellow colored material was obtained. The behavior of the recovered thymol, compared with the behavior of the original material, is shown below:

	Congealing Point	
	Original	Recovered
Seeded, after being supercooled to:		
30°	49.0°	43.8°
35°	49.2°	44.7°
40°	49.4°	45.6°

4. Any acids that are present in a thyme oil might interfere with the determination of thymol unless they are removed by a preliminary treatment. The free acids of two normal Spanish oils were separated; the amount present is too small to offer any appreciable interference.

TABLE III.—RECONSTITUTED THYME OILS

No.	Composition, % Thymol	Carvacrol	Nonphenols	$d_{25°}^{25°}$	$\alpha_D^{25°}$	$n_D^{20°}$	Sol. 25°	Phenol Content (Vol., %)	Relative Viscosity at 22°[a]
A0	0	50	50	0.928	−1°26′	1.5020	Clearly soluble in 2–2.5 vols. 70% alcohol and more	48.4	1.6
A1	5	45	50	0.928	−1°26′	1.5018		48.0	...
A2	10	40	50	0.927	−1°26′	1.5016		48.0	...
A3	15	35	50	0.927	−1°26′	1.5013		48.0	...
A4	20	30	50	0.927	−1°26′	1.5013		48.0	...
A5	25	25	50	0.927	−1°26′	1.5012		48.0	1.6
A6	30	20	50	0.927	−1°26′	1.5012		48.0	...
A7	35	15	50	0.926	−1°26′	1.5012		48.0	...
A8	40	10	50	0.926	−1°26′	1.5010		48.0	...
A9	45	5	50	0.926	−1°26′	1.5009		48.0	...
A10	50	0	50	0.926	−1°26′	1.5008		47.8	1.6

[a] Determined by means of a pipette; viscosity of distilled water = 1.00.

TABLE IV.—BEHAVIOR OF RECOVERED PHENOLS

No.	Original Composition, % Thymol	Carvacrol	Mixture Cooled to 0°	5°	10°	15°	20°	25°	30°	35°	40°	45°	50°
A0	0	100	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
A1	10	90	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
A2	20	80	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
A3	30	70	C.S. Note 4	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
A4	40	60	C.S. Note 4	C.S. Note 4	C.S. Notes 1 & 3	N.C	N.C	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
A5	50	50				C.S. Note 4	C.S. Note 1.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C
A6	60	40					C.I. C.P. = 23.2°	C.I. Note 1	N.C.	N.C.	N.C.	N.C.	N.C.
A7	70	30					C.I. C.P. = 26.7°	C.I. C.P. = 29.8°	C.S. Note 1	N.C.	N.C.	N.C.	N.C.
A8	80	20						C.I. C.P. = 33.0°	C.I. C.P. = 35.3°	C.I. C.P. = 36.4°	N.C.	N.C.	N.C.
A9	90	10							C.I. C.P. = 39.5°	C.I. C.P. = 40.3°	C.I. C.P. = 41.5°	N.C.	N.C.
A10	100	0							C.I. C.P. = 43.8°	C.I. C.P. = 44.7°	C.I. C.P. = 45.4°	C.S. Note 1	N.C.

Key: N.C. = no crystallization observed; C.I. = crystallizes immediately; C.S. = crystallizes slowly; C.P. = congealing point.
NOTE 1—No appreciable temperature rise observed.
NOTE 2—Mixture becomes turbid owing to the presence of thymol crystals.
NOTE 3—Mixture becomes hazy owing to the presence of thymol crystals.
NOTE 4—Temperature rise is slow; congealing point is not sharp and is not easily reproducible.

Approximately 100 cc. of thyme oil was shaken thoroughly with 25 cc. of sodium carbonate T.S.: no liberation of carbon dioxide was observed. After twenty-four hours, the aqueous layer was separated and washed with three 25-cc. portions of ether to remove any dissolved or suspended oil. After acidification with 25 cc. of dilute sulfuric acid (1:3), the liberated acids were extracted with 50 cc. ether. Upon evaporation of the dried ether layer, a small amount of a dark oily liquid with the

characteristic odor of the lower fatty acids was obtained. The amount recovered weighed 0.012 Gm. and 0.016 Gm. for the two thyme oils used in this experiment.

DISCUSSION

A study of Table II shows that a mixture of the two phenols containing 20% thymol will show no crystal formation within five minutes when cooled to 0° and seeded. The 30% thymol sample, however, crystallizes slowly at 0° as well as at 5°; no appreciable temperature rise is observed, however. The 50% thymol sample is the first to show a decided rise in temperature during the crystallization. In this case the congealing point is obviously dependent upon the degree of supercooling; when supercooled to 5°, the congealing point was 15.5°; when supercooled to 15°, the congealing point was 20.5°. As the percentage of thymol is increased from 50% to 100%, the congealing points become sharper and more easily reproducible. However, even the 100% thymol sample showed some variation in congealing point depending upon the degree of supercooling.

Sage and Dalton (7) reported values in disagreement with these data. Unfortunately, these authors did not give constants for the carvacrol used in their experiments; possibly the carvacrol contained thymol, since it is frequently obtained in commerce as a by-product in the manufacture of thymol. It is interesting to note that these investigators reported no difficulty was encountered in determining congealing points, even with mixtures containing but 10% thymol. Furthermore, no depression of congealing point was reported for pure carvacrol when admixed with small percentages of thymol.

In Table IV it will be seen that a convenient change in behavior occurs between 40% and 50% thymol; i.e., separated phenols consisting of 50% thymol crystallize at 20° (normally considered room temperature), but a temperature below 15° is required for crystallization of the separated phenols consisting of 40% thymol. Furthermore, this same relationship exists for mixtures of the pure phenols (see Table II).

The congealing points reported by Sage and Dalton (7) for recovered phenols are in disagreement with the data given in Table IV; again the possibility that their carvacrol contained thymol cannot be overlooked.

The greatest difference in behavior of the mixtures of pure phenols and the phenols recovered from the reconstituted thyme oils occurs at high thymol percentages. This is to be expected, since the melting point of a pure substance (thymol) is lowered considerably more by a small amount of impurity than is the melting point of a mixture (50% thymol–50% carvacrol). However, if some arbitrary value is selected as a criterion for a thymol type of oil, this factor will be minimized, provided that the arbitrary value is fixed at a low figure; for example, 50% thymol.

The procedure described requires the use of 25 cc. of oil. This amount was chosen so that approximately 10 Gm. of recovered phenol would be available for investigation. However, if such a quantity of oil is not available the aqueous alkaline solution from the official determination of total phenols may be used. The quantities of solutions and reagents employed should be reduced proportionately. The recovered phenols in such cases amount to approximately 4 to 6 Gm., depending on the total phenol content of the oil. The behavior on seeding at various fixed temperatures is in close agreement with the values reported for the 10-Gm. samples.

SUMMARY

A procedure has been described for the separation of phenols from thyme oils; the behavior of known mixtures of such separated phenols at fixed temperatures when seeded with a crystal of thymol has been described.

If the phenols separated from Spanish thyme oil crystallize at room temperature (20°) after seeding, the phenols of the original oil consist of more than 40% thymol; these oils cannot be of the carvacrol type. Such behavior is indicative that the oil is primarily of the thymol type, official in the National Formulary, Eighth Edition.

Furthermore, if the separated phenols crystallize within five minutes when cooled to 0° and seeded, the phenols of the original oil contain more than 20% thymol.

The experimental part of this paper follows the general outline of the work of Sage and Dalton (7); however, the results are at variance to the data presented in this earlier work. The discrepancies may well be due to the fact that the carvacrol employed by Sage and Dalton may have contained thymol as an impurity.

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Improved Methods of Tablet Coating*

By JOSEPH A. KOREN and BYRL E. BENTON

This paper describes an attempt to develop a standardized method of tablet coating; i.e., the application of well-defined procedures of precise quantities of liquids and solids to a definite weight or volume of tablets. Experiments presented show that the standardization of tablet coating is possible. The disintegration tests show that the tablets coated by the outlined processes disintegrate rapidly.

COATING of solid medicinals has been practiced for a long time and the operation has been regarded as one of the mystic arts (1). Stokes (2) and Chilson (3) point out that the processes and techniques employed in the coating of tablets have been surrounded with more or less secrecy. Tablet coating, according to Chilson (4), is one of the most variable processes in the pharmaceutical industry; it is wholly governed by rule-of-thumb methods and has in consequence led to a variety of formulas and methods. According to the information found in the literature, the general opinion seems to be that tablet coating is an "art" which can be acquired by practice and experience rather than by the use of accurate weights and measures and standard procedures.

The need for further study on tablet coating has been shown by the numerous and various methods now employed and by the lack of adequate information reported in the literature regarding a standard procedure. This paper describes an attempt to develop a standardized method of tablet coating; i.e., the application by well-defined procedures of precise quantities of liquids and solids to a definite weight and volume of tablets. It is especially important that the liquids and solids be combined in a definite ratio, thereby eliminating the guesswork in the most variable operation of tablet coating. The conventional methods were studied and improved methods were established; these methods were then applied and the results recorded.

EXPERIMENTAL

The equipment used in these investigations consisted of a 20-inch galvanized iron coating pan and a 20-inch canvas-lined galvanized iron polishing pan. These two pans were interchangeable on the same motor-driven bench stand. The motor was geared down so that the pan on the shaft made 36 r. p. m.

The hot air blast was supplied by the exhaust and the vacuum by the suction of a commercial vacuum cleaner. The hot air blast was used simultaneously with the vacuum at all times during the coating process. After the vacuum cleaner had been running for approximately twenty minutes the temperature of the hot air blast reached 55°. The hot air exhaust was directed at the upper end of the pan, while the suction end was placed slightly above the tablets. The position of the two ends created a constant air current, heated the pan, and removed free dust or powder that was given off by the tablets during the drying process.

A hot plate and a water bath supplied the heat necessary for heating the coating liquids during the coating operation.

A Waring Blendor with blades revolving at a speed of 10,000 r. p. m. was used for mixing all the suspensions before they were applied to the tablets. The glass container of the Waring Blendor was graduated so that the suspensions could be accurately measured.

The Hardness Tester for Tablets furnished by the Monsanto Chemical Company of St. Louis, Mo., was used to determine the degree of hardness of the tablets.

The disintegration of the tablets (uncoated and coated) was determined by an instrument employed by Granberg (5). A constant water bath with a temperature of 37° was used to maintain the acid-pepsin solution (6) at body temperature during the disintegration tests.

Three thousand nine hundred grams of four-grain placebo tablets (approximately 15,000 tablets) were employed in all the coating operations. The placebo tablets contained 10 parts of lactose and 1 part of starch which was mixed with $1^1/_2$ parts of starch paste (10% *w/v*) and granulated through a No. 12 sieve. After the granules were dry, 4% starch, 2% talc, and 0.5% magnesium stearate were added to facilitate the compression of the tablets. The tablets were compressed on a Stokes model "E" single-punch machine using deep concave $^{11}/_{32}$-inch punches and die. The pressure was adjusted so that the tablet hardness was between 3 and 4 Kg.

The following formulas were employed in these investigations (Table I). All of the ingredients are of U. S. P. or N. F. quality unless otherwise indicated.

The shellac solution used was the full strength Nu-Pros Glaze manufactured by James B. Day Company of Chicago, Ill. The shellac solution contains 4 lb. of arsenic-free shellac in sufficient alcohol to make one gallon.

The classification of the operations in the process of coating tablets varies among the different men

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with experience in coating. The coating employed in these experiments has been divided into the undercoat, the subcoat, the syrup coat, the finishing coat, and the polishing coat.

During the undercoating process, it was found that 150 cc. of the shellac solution was sufficient to give satisfactory results.

In the first experiment of the subcoating process the same amount of the subcoating solution (Formula 1) was added for each application (Table II). Sufficient powder (Formula 2) was then applied to the tablets to prevent excessive sticking. The powder was in a tared vessel which was weighed before and after each dusting. It was noticed that if a particular application required a great quantity of powder the next application would require less powder and vice versa. An analysis of the quantities of dusting powder used with a constant amount of subcoating solution indicated that there was a relationship between the amount of subcoating solution and the amount of dusting powder.

In the next two experiments, the quantity of the subcoating solution was reduced after the first application and then kept constant in subsequent applications. It was decided to decrease gradually the amount of the dusting powder to avoid the accumulation of an excessive amount of free powder.

During the first three experiments it was noticed that the amount of dusting powder required to prevent the tablets from becoming excessively sticky also depended upon when the powder was added to the tablets.

To eliminate the guesswork as to when to add the powder and how much powder to add for a smoother subcoat and to reduce the time required for tablet coating, the dusting powder was incorporated in the subcoating solution in the fourth experiment. Various quantities of this subcoating suspension (Formula 3) were applied.

The results obtained in the fourth experiment indicate that a combination of dusting powder and subcoating solution applied as a suspension gives satisfactory results.

TABLE I.—PERCENTAGE COMPOSITION OF THE FORMULAS EMPLOYED IN TABLET COATING

Ingredient	1	2	3	4	5	6	7	8	9	10	11	12	13
Gelatin	3.5		..	10	5	.	3						
Acacia	3	4	.	10	5	.	3						
Sugar	55	72	.	50	50	85							
Talc	..	4	..	10	25			5	10	5			
Starch	.	20	.	10	5			15	30	15			
Formula 1	..		50										
Formula 2	..	.	50										
Formula 6	..			.			94		60	80	50		
Formula 7	..	.	..	.		..	.	80					
White wax	...		.	.	..	.		..	.	..	..	67	
Carnauba wax	..						.	..	.	..	.	33	
Formula 12							.	..	..	..	..	.	5
Naphtha (commercial grade)	..			.	.	.				..	..	..	95
Dist. water	38 5	.	.	10	10	15	..	.	..	..	50		
Total	100	100	100	100	100	100	100	100	100	100	100	100	100

Formulas 1, 3, 4, and 5 were applied at a temperature of 70°. Formula 2 was sifted through a No. 80 screen. Formulas 6, 7, 9, 10, and 11 were applied at a temperature of 20°. Formulas 8 and 13 were applied at a temperature of 50°. Formula 12 was melted, mixed well, and allowed to solidify.

TABLE II.—QUANTITIES APPLIED DURING THE SUBCOATING PROCESS

Experiment	Formula Used	Application[a] 1	2	3	4	5	6	7	8	9	10	11	12
1	1	75	75	75	75	75	75	75					
	2	30	15	60	20	40	35	40					
2	1	75	50	50	50	50	50	50	50				
	2	60	30	30	15	15	15	15	30[b]				
3	1	60	50	50	50	50	50	50	50	50	50		
	2	40	35	30	25	25	25	20	20	15	0		
4	3	60	120	100	75	75	75	120	75	75	75		
5	4	100	60	25	40	15	20						
	5	..	..	..	..	..	..	40	60	65	60	60	80
6	8	60	50	50	60	75	100	125	150	200			
7	8	50	65	80	125	150							
8	8	60	100	150	175	150	150	150					
9	10	60											
	8	..	50	75	100	125	150	150	175	175	175	200	
10	9	60											
	8	..	75	75	100	125	150	150	150	175	175	200	175

[a] Solids weighed in grams. Liquids measured in cubic centimeters.
[b] The amount of the dusting powder was increased because the tablets were not filling out.

In an attempt to obtain a more favorable ratio between the powder and the liquid, the subcoating mixture No. 1 (Formula 4) and the subcoating mixture No. 2 (Formula 5) were employed in the fifth experiment.

The results obtained from the applications of the subcoating mixture No. 1 indicated that too much adhesive substances and not enough insoluble powders were present; therefore, subcoating mixture No. 2 was formulated.

Since the subcoating mixtures Nos. 1 and 2 employed in the fifth experiment were unsatisfactory a new compound subcoating mixture (Formula 8) was employed in the sixth experiment. With the exception of the ninth application, gradual increase of the amount of the mixture gave satisfactory results. Too much of the mixture was added in the ninth application and the tablets became excessively sticky. The compound subcoating mixture gave satisfactory results until an excess amount was added. It was therefore decided to employ it in the seventh experiment.

As in the previous experiment, the first application of the compound subcoating mixture (Formula 8) in the seventh experiment (even though the quantity was reduced) caused the tablets to stick to the side of the pan. However, when the second application was added, although the amount was increased, the tablets did not become excessively sticky. In fact, all of the subsequent applications gave satisfactory results. It may be possible that the excessive sticking after the first application may be due to the shellac undercoat plus the moisture content of the subcoating liquid. To test this hypothesis the undercoat was omitted in the eighth experiment and the compound subcoating mixture was applied directly to the tablets. The tablets did not stick excessively; however, they did become tacky and had pinholes in them where they had pulled apart from each other. The amount of the compound subcoating mixture was increased in the next two applications without any excessive sticking, but the amount of the mixture in the fourth application caused the tablets to stick excessively. The sticking after the fourth application was due to the addition of an excessive amount of the mixture. The amount of the mixture was reduced in the subsequent applications and satisfactory results were obtained.

The undercoat was applied as usual in the ninth experiment because it was found to give a better foundation for subsequent coats. To prevent excessive sticking when the first application of the compound subcoating mixture was applied, the syrup mixture No. 2 (Formula 10) was employed after the undercoat. The syrup mixture No. 2 made the tablets very tacky; however, the tablets did not stick to the side of the pan. The first application of the compound subcoating mixture did not cause the tablets to stick excessively; on the contrary, the tablets became only slightly tacky. The amount of the compound subcoating mixture was gradually increased in the subsequent applications and in each case the action of the tablets was satisfactory. These results indicate that the application of a syrup mixture before subcoating a tablet may be desirable.

Although the tablets did not stick to the side of the pan when the syrup mixture No. 2 was applied in the ninth experiment, they did become very tacky. Therefore, a priming mixture (Formula 9) was formulated and employed in the tenth experiment. This mixture was applied after the undercoat. There was no excessive sticking either with the priming mixture or with the first application of the compound subcoating mixture. The quantity of the compound subcoating mixture was gradually increased in the subsequent applications until the eleventh application. The tablets became excessively sticky after the eleventh application; therefore, the amount was decreased in the last application.

Various amounts of syrup solution (Formula 6) and syrup mixtures (Formula 10) (Table II) were applied in each experiment in an attempt to obtain the proper quantity to be used for a definite weight of tablets. It was found that 60 cc. of syrup mixture No. 2 and 40 cc. of syrup solution gave satisfactory results.

During the finishing process it was found that 15 or 20 cc. of the finishing solution (Formula 11) could be applied to the tablets without causing them to stick excessively.

During the polishing process it was found that one application of 60 cc. of the wax solution (Formula 13) was sufficient to impart a satisfactory luster to the tablets.

Several *in vitro* methods have been suggested for determining the relative rate of disintegration of the tablets. The procedure employed by Granberg (5) was selected for this study. Ten uncoated and ten coated tablets from each experiment were tested to give an average disintegrating time (Table III).

TABLE III.—AVERAGE DISINTEGRATION TIME IN MINUTES AND SECONDS

Experiment	Uncoated Tablet	Coated Tablet
1	13 sec.	9 min., 35 sec.
2	14 sec.	8 min., 34 sec.
3	12 sec	2 min., 50 sec.
4	10 sec.	3 min., 41 sec.
5	10 sec.	2 min., 37 sec.
6	12 sec.	2 min., 35 sec.
7	12 sec.	1 min., 30 sec.
8	12 sec.	2 min., 18 sec.
9	12 sec.	2 min., 32 sec.
10	29 sec.	4 min., 10 sec.

SUMMARY AND CONCLUSION

1. An undercoat of shellac is desirable as a foundation for subsequent coatings to produce smooth tablets.

2. A priming coat consisting of insoluble powders suspended in syrup solution aids in producing a smooth tablet.

3. The incorporation of insoluble powders in the subcoating solution facilitates their application to the tablet and at the same time produces a smoother subcoat.

4. The amounts of syrup mixture, syrup solution, and finishing solution have been standardized for a given weight of tablets.

5. Tests show that the tablets coated by the outlined processes disintegrate rapidly.

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A Contribution to the Pharmacology of 5-Nitroguaiacol*,†

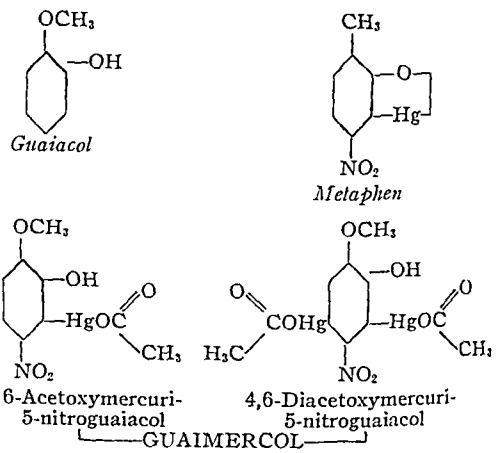
By NORMAN W. PINSCHMIDT‡ and JOHN C. KRANTZ, JR.

***In vitro* tests on mercurated 5-nitroguaiacol indicate that it may be an effective antiseptic, both in its solid form and in solution as the sodium salt. The results of toxicity studies are reported.**

THE ORGANIC mercurial antiseptics are, for the most part, monomercury substitution products of benzene and its derivatives. In a study concerned with the mercuration of the benzene nucleus certain derivatives of guaiacol were made available. Of the many mercury compounds of guaiacol and its derivatives which were synthesized in this study, the compound obtained by direct mercuration of 5-nitroguaiacol appeared to be the most promising for pharmacologic study. Owing to the former widespread empirical use of guaiacol in pulmonary infections, we decided to investigate this compound.

In a study of the structure of these mercury derivatives of 5-nitroguaiacol, Drake, Harris, and Jaeger (1) have shown that direct mercuration of 5-nitroguaiacol with mercuric acetate results in a product having a mercury content of 49 to 54 per cent (depending upon the exact mercuration conditions) and that it consists of, principally, 6-acetoxymercuri-5-nitroguaiacol and 4,6-diacetoxymercuri-5-nitroguaiacol. The product is very insoluble in water and organic solvents, with the exception of acetic acid, and does not have a clearly defined melting point. It has resisted every effort to separate it into pure components. It is available under the name of "Guaimercol."

The acetoxymercuric derivative of 5-nitroguaiacol used in this study is a yellow, finely divided powder fluorescing as brick-red under ultraviolet light. It has a mercury content of 53.8 per cent. Its relationship to guaiacol and "Metaphen" is apparent from the following formulas.



Strong bases form the corresponding soluble phenolates of the compound. Each 100 mg. of the powder is therefore treated with 2 cc. of 1 *N* sodium hydroxide before dilution with water. The 1:1000 solution, thus prepared, although strongly alkaline (*p*H 10.5 to 11) is suitable for intravenous injection if administered slowly. Irrespective of treatment by various buffers and protective colloids, solutions of Guaimercol are unstable at a *p*H below 10.5. The alkaline solution is unstable in air but if preserved under nitrogen in sealed glass ampules, it will resist extreme conditions of light, temperature, and agitation.

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TOXICITY STUDIES

In establishing the values given in Table I, the method of Behrens (2) for deduced mortalities was used, the LD_{50} being obtained by graphing.

In the case of the intraperitoneal injection in rats a comparison has been made between young and older animals. The factors of weight and age seem to have little effect in the toxicity of this compound.

Representative animals of the higher dose ranges used in establishing the LD_{50}'s were autopsied and microscopic studies were made on their tissues. The typical lesions of mercurial poisoning were found in the kidneys and colon. In addition, some subcapsular necrosis was found in the liver. All other organs appeared normal.

In order to establish the chronic or cumulative toxicity, the compound was administered in one-half the minimum lethal dose (0.5 mg./Kg.) to a series of five rabbits. The drug was given intravenously twice weekly for a period of six weeks. At the end of this period the animals were sacrificed and liver, kidney, and colon sections were examined microscopically. In no instance was there any symptom of mercurial poisoning other than a slight cloudy swelling of the proximal tubules of the kidney. Two of the animals developed late in the study a severe diarrhea and loss of weight and finally expired. Their pathology, however, did not in any way resemble that of mercurial poisoning, one apparently dying of an infection of the large bile ducts and the other of unknown causes. The other animals remained healthy and normal throughout the experiment and showed a normal weight increase.

A further study of the cumulative toxicity was made on a series of 12 dogs to which was administered an intravenous dose of 0.5 mg./Kg. on five successive days. The animals were biopsied in from three to five days following the last injection and histological examinations made of their livers and kidneys. With the exception of a mild swelling of the tubules with little or no debris in the lumina, the kidneys were normal. The livers showed in several cases a cloudy swelling of a diffused nature and in one case some slight degeneration of the liver cells. As those changes are reversible and of such a mild nature, they are not considered significant of chronic irreparable damage.

Bromsulfonphthalein Test for Liver Function.—Six dogs were tested for normal liver function according to the standard values used in this laboratory, i.e., that less than 3% of the dye remains in the blood stream thirty minutes after injection.

The animals were then injected intravenously on each of five successive days with 0.5 mg./Kg. Guaimercol. After two days the bromsulfonphthalein test was repeated for three successive days on each animal. No deviation from the normal was observed.

Phenolsulfonphthalein Test for Kidney Function.—Six dogs were injected for five days with an intravenous dose of 0.5 mg./Kg. of Guaimercol.

After two days rest the dye was administered by intramuscular injection and overnight (eighteen hours) urine samples collected. These samples showed an elimination of the dye in amounts comparable to values on normal dogs.

In order to confirm these findings, the dogs were further tested at the time of the biopsy for the collection of kidney and liver specimens (above). After the removal of one kidney under pentobarbital anesthesia, the ureter of the remaining kidney was cannulated and the urine flow adjusted, by means of intravenous injection of 50% glucose, to from 7 to 10 drops per minute. Urine was collected over a period of two and one-half hours and made up to 500 cc. for comparisons with U. S. P. standards. The values obtained (45 to 55% dose) compared favorably with those obtained on normal dogs by the same method.

Reaction with Normal Body Constituents.—Tests were run with Guaimercol against a representative sulfur-bearing compound, cysteine hydrochloride. Solutions of Guaimercol 1:1000 were apparently unaffected even upon long standing (seventy-two hours) by an equal volume of cysteine hydrochloride solution 1:1000.

Rabbit, horse, and human serum in concentrations ranging from 10 to 50% were mixed with Guaimercol 1:1000, Mercurochrome 1:50, Metaphen 1:1000, and mercuric chloride 1:1000 and allowed to stand at 37° for twenty-four hours. Only in the case of mercuric chloride was there any great degree of precipitation. The three organic mercurials showed no greater precipitation of serum than was caused by an equal volume of distilled water.

Guaimercol and mercurochrome were found to be soluble in normal horse serum.

Respiration Studies.—Studies on Guaimercol were conducted by Dr. Wilson C. Grant of this laboratory using the Warburg technique for the study of the respiration of isolated tissue sections.

The mercurated 5-nitroguaiacol compound in the concentration employed caused a depression in the respiratory quotient of kidney and tumor tissue only.

It may be calculated that the concentration of mercurated 5-nitroguaiacol used in this test is ap-

TABLE I.—COMPARATIVE TOXICITY OF GUAIMERCOL WITH VARIOUS MODES OF ADMINISTRATION: COMPARATIVE TOXICITY WITH METAPHEN

Compound	Animal	No. Animals	Wt., Gm.	Mode of Administration	LD_{50} (Mg./Kg.)
Guaimercol	Albino rats	42	140–160	Intraperitoneally	6.0
	Albino rats	36	180–210	Intraperitoneally	6.9
	Albino rats	54	180–250	Orally	125.0
	Rabbits	42	1500–2400	Intravenously	2.6
Metaphen	Albino rats	36	140–160	Intraperitoneally	3.0

5. Tests show that the tablets coated by the outlined processes disintegrate rapidly.

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A Contribution to the Pharmacology of 5-Nitroguaiacol*,†

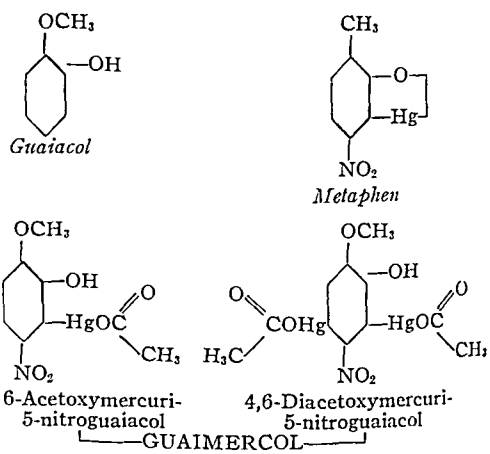
By NORMAN W. PINSCHMIDT‡ and JOHN C. KRANTZ, JR.

***In vitro* tests on mercurated 5-nitroguaiacol indicate that it may be an effective antiseptic, both in its solid form and in solution as the sodium salt. The results of toxicity studies are reported.**

THE ORGANIC mercurial antiseptics are, for the most part, monomercury substitution products of benzene and its derivatives. In a study concerned with the mercuration of the benzene nucleus certain derivatives of guaiacol were made available. Of the many mercury compounds of guaiacol and its derivatives which were synthesized in this study, the compound obtained by direct mercuration of 5-nitroguaiacol appeared to be the most promising for pharmacologic study. Owing to the former widespread empirical use of guaiacol in pulmonary infections, we decided to investigate this compound.

In a study of the structure of these mercury derivatives of 5-nitroguaiacol, Drake, Harris, and Jaeger (1) have shown that direct mercuration of 5-nitroguaiacol with mercuric acetate results in a product having a mercury content of 49 to 54 per cent (depending upon the exact mercuration conditions) and that it consists of, principally, 6-acetoxymercuri-5-nitroguaiacol and 4,6-diacetoxymercuri-5-nitroguaiacol. The product is very insoluble in water and organic solvents, with the exception of acetic acid, and does not have a clearly defined melting point. It has resisted every effort to separate it into pure components. It is available under the name of "Guaimercol."

The acetoxymercuric derivative of 5-nitroguaiacol used in this study is a yellow, finely divided powder fluorescing as brick-red under ultraviolet light. It has a mercury content of 53.8 per cent. Its relationship to guaiacol and "Metaphen" is apparent from the following formulas.



Strong bases form the corresponding soluble phenolates of the compound. Each 100 mg. of the powder is therefore treated with 2 cc. of 1 *N* sodium hydroxide before dilution with water. The 1:1000 solution, thus prepared, although strongly alkaline (*p*H 10.5 to 11) is suitable for intravenous injection if administered slowly. Irrespective of treatment by various buffers and protective colloids, solutions of Guaimercol are unstable at a *p*H below 10.5. The alkaline solution is unstable in air but if preserved under nitrogen in sealed glass ampules, it will resist extreme conditions of light, temperature, and agitation.

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† This work was supported by a grant from The Guaimer Laboratories, Inc., Kansas City, Mo.
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TOXICITY STUDIES

In establishing the values given in Table I, the method of Behrens (2) for deduced mortalities was used, the LD_{50} being obtained by graphing.

In the case of the intraperitoneal injection in rats a comparison has been made between young and older animals. The factors of weight and age seem to have little effect in the toxicity of this compound.

Representative animals of the higher dose ranges used in establishing the LD_{50}'s were autopsied and microscopic studies were made on their tissues. The typical lesions of mercurial poisoning were found in the kidneys and colon. In addition, some subcapsular necrosis was found in the liver. All other organs appeared normal.

In order to establish the chronic or cumulative toxicity, the compound was administered in one-half the minimum lethal dose (0.5 mg./Kg.) to a series of five rabbits. The drug was given intravenously twice weekly for a period of six weeks. At the end of this period the animals were sacrificed and liver, kidney, and colon sections were examined microscopically. In no instance was there any symptom of mercurial poisoning other than a slight cloudy swelling of the proximal tubules of the kidney. Two of the animals developed late in the study a severe diarrhea and loss of weight and finally expired. Their pathology, however, did not in any way resemble that of mercurial poisoning, one apparently dying of an infection of the large bile ducts and the other of unknown causes. The other animals remained healthy and normal throughout the experiment and showed a normal weight increase.

A further study of the cumulative toxicity was made on a series of 12 dogs to which was administered an intravenous dose of 0.5 mg./Kg. on five successive days. The animals were biopsied in from three to five days following the last injection and histological examinations made of their livers and kidneys. With the exception of a mild swelling of the tubules with little or no debris in the lumina, the kidneys were normal. The livers showed in several cases a cloudy swelling of a diffused nature and in one case some slight degeneration of the liver cells. As those changes are reversible and of such a mild nature, they are not considered significant of chronic irreparable damage.

Bromsulfonphthalein Test for Liver Function.—Six dogs were tested for normal liver function according to the standard values used in this laboratory, i.e., that less than 3% of the dye remains in the blood stream thirty minutes after injection.

The animals were then injected intravenously on each of five successive days with 0.5 mg./Kg. Guaimercol. After two days the bromsulfonphthalein test was repeated for three successive days on each animal. No deviation from the normal was observed.

Phenolsulfonphthalein Test for Kidney Function.—Six dogs were injected for five days with an intravenous dose of 0.5 mg./Kg. of Guaimercol.

After two days rest the dye was administered by intramuscular injection and overnight (eighteen hours) urine samples collected. These samples showed an elimination of the dye in amounts comparable to values on normal dogs.

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In order to confirm these findings, the dogs were further tested at the time of the biopsy for the collection of kidney and liver specimens (above). After the removal of one kidney under pentobarbital anesthesia, the ureter of the remaining kidney was cannulated and the urine flow adjusted, by means of intravenous injection of 50% glucose, to from 7 to 10 drops per minute. Urine was collected over a period of two and one-half hours and made up to 500 cc. for comparisons with U. S. P. standards. The values obtained (45 to 55% dose) compared favorably with those obtained on normal dogs by the same method.

Reaction with Normal Body Constituents.—Tests were run with Guaimercol against a representative sulfur-bearing compound, cysteine hydrochloride. Solutions of Guaimercol 1:1000 were apparently unaffected even upon long standing (seventy-two hours) by an equal volume of cysteine hydrochloride solution 1:1000.

Rabbit, horse, and human serum in concentrations ranging from 10 to 50% were mixed with Guaimercol 1:1000, Mercurochrome 1:50, Metaphen 1:1000, and mercuric chloride 1:1000 and allowed to stand at 37° for twenty-four hours. Only in the case of mercuric chloride was there any great degree of precipitation. The three organic mercurials showed no greater precipitation of serum than was caused by an equal volume of distilled water.

Guaimercol and mercurochrome were found to be soluble in normal horse serum.

Respiration Studies.—Studies on Guaimercol were conducted by Dr. Wilson C. Grant of this laboratory using the Warburg technique for the study of the respiration of isolated tissue sections.

The mercurated 5-nitroguaiacol compound in the concentration employed caused a depression in the respiratory quotient of kidney and tumor tissue only.

It may be calculated that the concentration of mercurated 5-nitroguaiacol used in this test is ap-

proximately that which would appear in the blood of an animal receiving a dose of 6.6 mg./Kg. of the drug. Reference to the toxicity table previously given shows this to be a fatal dose (corresponding with the LD_{50} in rats).

PHARMACODYNAMIC STUDIES

Blood Pressure.—Stefano and Querico (3) have shown that guaiacol injected intravenously into higher animals is a blood pressure reductant. Guaimercol, however, did not alter the blood pressure of dogs under ether anesthesia at any of a wide range of doses. As much as 30 cc. of a 1:500 alkaline solution failed to produce any change in 5 medium-sized dogs injected

Cardiac Toxicity.—It has also been shown by Stefano and Querico that guaiacol causes a depression of the isolated frog's heart that is sometimes irreversible. In the frog heart perfusion (thirteen minutes) with a 1:100,000 solution of mercurated 5-nitroguaiacol in Howell-Ringer's solution, there was no change in amplitude, rhythm, or time of the heart beat.

Diuresis.—A series of six dogs was studied in which the ureters were cannulated and the urine flow measured before and after administration of large doses (10 cc. of 1:1000 alkaline solution to 8 to 10 Kg. dogs). In no case was there any significant increase in the flow of urine.

Bronchiolar Action.—The compound was perfused through the freshly isolated lungs of the guinea pig. The degree of bronchiolar dilatation or constriction was measured by counting the number of drops of saline and drug as they perfused through the organ. No significant change was observed in any of the preparations after addition of Guaimercol 1:1000 and 1:10,000 to the perfusion solution.

BACTERIOLOGICAL CONSIDERATIONS

The antiseptic potency of Guaimercol was established using the simplified Kolmer technique (4).

A $HgCl_2$ coefficient was established by dividing the denominator of the highest dilution of Guaimercol producing inhibition of each organism with the corresponding value for mercuric chloride. The results are shown in Table II.

TABLE II.—MERCURATED 5-NITROGUAIACOL VS. PATHOGENIC ORGANISMS (RESULTS AFTER FIVE DAYS OF INCUBATION)

Organism	Protection in Dilutions	$HgCl_2$ Coeff.
Staphylococcus aureus	Up to 1:960,000	27 3
Eberthella typhi	Up to 1:160,000	2.0
Flexnor bacillus	Up to 1:320,000	1.0
B. coli	Up to 240,000	0.69

Reaction with Body Fluids.—In order to approximate the action of the compound under conditions similar to those in living tissues, the antiseptic was similarly tested in varying dilutions with solutions containing 10% normal serum, 10% ascitic fluid, and 10% whole blood. The test organism was the standard *E. typhi* strain. With Guaimercol and three other organic mercurials studied, the range of protection was reduced to half by the presence of serum and ascitic fluid. The mercuric chloride was almost completely inactivated by the presence of these fluids.

With 10% serum we observed a very slight growth even in the highest concentrations attainable of all the mercurials. As this growth did not increase on standing and as 3 drops of solution plated on agar showed, in every case, less than 50 colonies, it was interpreted that there was a partial killing and bacteriostasis of the organism in the presence of serum.

In the presence of a 10% defibrinated human blood the mercurated 5-nitroguaiacol showed no bacteriostasis in concentrations up to 1:10,000 (a relatively high concentration in terms of the normal potency of the compound).

Cup and Pour Plate Tests.—Using the standard cup and pour plate methods (with *E. Typhosa* and *S. Aureus*), there was observed a large area of clearing with the mercurated 5-nitroguaiacol. Of particular interest is the fact that the compound in its solid form, notwithstanding the fact that it is so insoluble in water, was toxic to organisms within a radius of 2 to 5 cm.

CONCLUSIONS

1. Some LD_{50}'s have been established for mercurated 5-nitroguaiacol which consists of, principally, 6-acetoxymercuri-5-nitroguaiacol and 4,6-diacetoxymercuri-5-nitroguaiacol. The compound is least toxic when administered by mouth.

2. Mercurated 5-nitroguaiacol has been shown to be administrable by vein in repeated sublethal doses without significant tissue damage.

3. When administered in lethal doses, it has been shown to produce damage primarily in the kidneys, colon, and liver, death apparently ensuing as a result of typical mercurial nephrosis. Lethal doses also produce an inhibition to the respiration of kidney and tumor.

4. *In vitro* tests show it to be an effective antiseptic of the order of 4-nitro-5-hydroxymercuriorthocresol (Metaphen-Abbott). It exhibits antiseptic properties both in its solid form and as the soluble sodium salt.

5. It has been shown to be without effect on blood pressure, heart action, urine output, or bronchiolar tone of animals.

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Preliminary Report On Microamorphous Silica as a Suspending Agent in Calamine Lotion*

By C. LEE HUYCK†

Two and four per cent aqueous suspensions of microcrystalline silica are described. These suspensions were substituted for 5 per cent aqueous bentonite suspension in Calamine Lotion and Chalk Mixture. Four per cent silica suspension appears to be superior to 5 per cent bentonite suspension in Calamine Lotion. Substitution of 5 per cent bentonite by 4 per cent silica suspension in Chalk Mixture proved to be unsatisfactory.

SPECIAL SOAPS containing silica (1) are made by treating a soap containing sodium silicate with a solution of phosphoric acid.

Silikat-Hautsalbe (2) is an ointment of lanolin and silicic acid used for frostbite, skin eruptions, pimples, red nose and face. Silikat-Milchpuder is a dusting powder containing silicic acid in an easily absorbed form indicated for wound, burn, skin itch, wet eczema, and inflammation.

Brock (3), *et al.*, prepared a silica gel suitable for cosmetic preparation by introducing silicon tetrafluoride into an aqueous solution of fluosilicic acid. Heuser used this form of silica gel as a dentifrice.

Leone and Sante (4) reported on the toxicity of pure, stable 0.5 per cent colloidal silica. When administered parenterally, colloidal silica exerts a depressing action in small doses and a paralyzing action at high doses.

Silantox (5) is a colloidal silica used internally as an intestinal absorbent and externally as a dusting powder.

Peronnet and Genet (6) prepared silica gel from sodium silicate and hydrochloric acid. It was filtered on a Buchner filter, washed and mixed with glycerin to give a gel having a consistency of petrolatum. Formulas are given for utilizing this gel as an ointment base. The ointment may be washed from the skin with water. No report is made on its therapeutic efficacy.

Belcot (7) published formulas of silica gel containing cosmetics and medicinal preparations.

Prout, Eddleman and Harris (8) investigated the value of such a base as a carrier for germicidal substances. They found that zinc oxide, boric acid, phenol and ammoniated mercury in a silica gel-glycerin base had greater zones of inhibition than the ointments of germicidal substances official in the United States Pharmacopoeia. The true value of this base, however, must await clinical evaluation.

Prout and Harris (9) reported on the emulsifying properties of silica gel and formulated a base consisting of 45 per cent silica gel, 5 per cent hydrous wool fat, 15 per cent liquid petroleum, and 35 per cent petrolatum.

Herfurth and Zellwalle (10) found that an emulsion of viscous petroleum oil and silica gel is suitable for use in ointments, creams, emollients, and soap mixtures.

Redgrove (11) referred to a patent by Heuser in which light amorphous silica is used to make excellent face powder bases These contain about 20 per cent silica. In addition to being light, and fluffy, the silica possesses excellent covering power and a remarkable capacity for absorbing moisture. In the two latter respects it is definitely superior to kaolin and precipitated chalk. While some felt that the silica might irritate the skin, the author felt that the use of colloidal or amorphous silica is without danger. In powder creams, amorphous silica gave exceptionally good results but was unsuitable in liquid powders because of its tendency to form hard gels.

EXPERIMENTAL

Microamorphous silica,[1] of a maximum particle size of 50 mμ was used. It has a linseed oil absorption value of 400, density of 2.2, index of refraction 1.46, purity of 99.9% and 8% absorbed water. Two and 4% aqueous suspensions were prepared by heating distilled water to boiling and adding the silica gradually with constant stirring. These suspensions were allowed to stand for twenty-four hours and then stirred vigorously again. The suspensions were substituted for 5% aqueous bentonite suspension in two pharmaceutical preparations requiring suspending agents, namely Calamine Lotion, and Chalk Mixture of the U. S. P. When 5% bentonite suspension was used in Calamine Lotion the average amount of water which separated on the surface of duplicate samples after forty-eight hours was 15 cc. per 85 cc. of finished product.

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† Director of the Department of Pharmacy, Howard College, Birmingham.

[1] Silica was furnished through the courtesy of the Linde Air Products Co., 30 E. 42nd St., New York 17, N. Y.

When 2% silica suspension was used as a suspending agent the average amount of water which separated from duplicate samples was 20 cc. per 90 cc. of finished product. When 4% silica suspension was substituted for the bentonite suspension the average amount of water which separated on the surface of duplicate samples was 7 cc. per 92 cc. of finished product. In Calamine Lotion, 4% silica suspension appeared to be superior to 5% bentonite suspension. When 4% silica suspension was substituted for 5% bentonite suspension in Chalk Mixture of the U. S. P., the colloidal characteristics of the product were immediately destroyed, i.e., separation of 80 cc. of water/95 cc. of finished product. With the purity of the chalk as a factor, precipitated calcium carbonate of the U. S. P. was substituted for the prepared chalk with the result that 60 cc. of water/95 cc. of finished product separated. The *p*H of Magma Bentonite U. S. P. was 8.4 while the *p*H of a freshly prepared 4% silica suspension was 4.95

DISCUSSION

Calcium carbonate appears to be a positively charged particle while SiO_2 appears to be a negatively charged particle; therefore the chalk mixture sol is coagulated by silicon dioxide. In the Calamine Lotion the calamine is a positively charged sol while the silica is negatively charged sol but no coagulation takes place. This may be due to preferential adsorption of the charges on the surface of particles; more important, however, is the fact that the solubility product of zinc hydroxide is about one-half the solubility product of calcium carbonate. The negative charge of the silicon dioxide would be immediately neutralized by th positive charge of the calcium carbonate in solutio causing coagulation and precipitation of calciu silicate.

SUMMARY

1. Four per cent microcrystalline silic appears to be superior to five per cent bentonit magma in Calamine Lotion of the U. S. P.

2. Four per cent microcrystalline silica ap pears to be inferior to 5 per cent bentonite magm in Chalk Mixture of the U. S. P.

3. Reasons are advanced for the success an failure of this material as a suspending agent.

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The Effects of B Vitamins, Liver, and Yeast on Promin Toxicity in the Rat*

By B. H. ERSHOFF and H. B. McWILLIAMS†

Toxic effects resulting from the administration of massive doses of promin were largely counteracted in rats by the administration of desiccated whole liver. The protective factor was distinct from any of the known vitamins and was not present in the casein or salt mixture employed. The suggestion is mad ethat many of the toxic effects observed in animals fed massive doses of certain drugs are in reality due to a deficiency of an unknown nutrient(s) resulting from an increased requirement following drug administration. Desiccated whole liver is a rich source of this unknown factor(s).

PROMIN (sodium *p,p'*-diaminodiphenylsulfone-N,N'-didextrose sulfonate) has been employed experimentally in the treatment of tuberculosis, leprosy, and infections caused by th hemolytic streptococcus, the pneumococcus, an the gonococcus (1–5). Toxic reactions followin the administration of promin have discourage the use of this drug, particularly with the adven of promizole, a related sulfone, considerably les toxic than promin, and also of value in the treat ment of experimental tuberculosis (6, 7). Toxi effects of promin administration may be counter acted, however, to a large extent by dietary

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† We are indebted to Dr. L. A. Sweet of Parke, Davis and Co., Detroit, Mich., for the promin employed in the present experiment.

means. Higgins (8) observed that an increased intake of thiamine, riboflavin, and pyridoxine exerts a protective effect in animals receiving massive doses of this drug. More recently Higgins (9) found that toxic effects might be controlled further by the administration of whole liver or yeast. In the present communication further data are presented on the effects of diet on promin toxicity in the rat.

PROCEDURE AND RESULTS

Four basal rations were employed in the present experiment: diets A, B, C, and D. Diets A and B were purified rations containing the B complex factors in synthetic form only. Diets C and D were similar in composition but contained yeast or desiccated whole liver in addition to the synthetic vitamins. All four rations were supplemented with 0.0 or 5.0 Gm. of promin (sodium *p,p'*-diaminodiphenylsulfone-N,N'-didextrose sulfonate) per Kg. of diet. In addition to the above, two further rations (diets E and F) were employed, similar to diet D but containing the water-soluble extract and the water-insoluble residue of liver, respectively, in place of the whole liver. Eighty-four female rats of the Sprague-Dawley strain were selected at twenty-one to twenty-three days of age and an average weight of 44.2 Gm. for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces (two animals per cage); and were fed *ad lib.* the diets listed in Table I. Feeding was continued for eight weeks or until death, whichever occurred sooner. Animals were autopsied on the fifty-sixth day of feeding, and thyroid, ovarian, and adrenal weights determined. Results are summarized in Table II.

Findings confirm the earlier work of Higgins (9) that toxic effects of promin administration may be counteracted in the rat by dietary means. Toxic effects were most pronounced on the synthetic ration (diet A_1) and least evident on the ration containing extracted liver residue (diet F_1). Growth was retarded in all promin-fed rats. Gain in body weight was greater, however, on diets D_1, E_1, and F_1 than on other promin-containing rations. Growth was particularly marked in animals fed the extracted liver residue (diet F_1).[1] On promin-free diets no significant difference in growth was observed on any of the rations employed. The effects of promin administration were most pronounced during the first week of feeding. During this period a considerable number of animals lost weight, particularly those on synthetic rations (diets A_1 and B_1). Forty per cent of the rats on diet A_1 and 30% of those on diet B_1 died at this time. A marked hyperirritability of all promin-fed rats was noted, particularly during the first four days of feeding. Animals were observed chewing each others ears and tail and upper back; and a considerable loss of hair occurred, particularly on the synthetic rations (diets A_1 and B_1). After the first week of feeding growth was resumed in all groups and a marked reduction of irritability was noted.

The protective effect of extracted liver residue in promin-fed rats was similarly noted in respect to gonadal weight. Ovarian development was markedly inhibited in promin-fed rats on the synthetic rations (diet A_1 and B_1) and the ration containing yeast (diet C_1). Ovaries were somewhat larger on diets D_1 and E_1 containing whole liver and water-soluble extract of liver, respectively. Ovarian weights were virtually normal, however, in promin-fed rats receiving extracted liver residue (diet F_1). No significant difference in ovarian weight was noted on promin-free diets on any of the rations employed (Table II).

A marked goitrogenic effect was observed following promin administration on all diets employed. Thyroid weights on promin-containing rations averaged 33.5 to 60.7 mg. per 100 Gm. body weight in contrast to values of 5.5 to 6.6 mg. per 100 Gm. body weight on promin-free rations (Table II). No correlation was obtained, however, between the extent of thyroid enlargement and other manifestations of promin toxicity.

A significant difference in adrenal weight was observed in promin-fed rats on the various rations (Table II). The adrenal weight of promin-fed rats receiving extracted liver residue (diet F_1) was significantly greater than that of promin-fed rats on other diets with the possible exception of diet D_1. When expressed in terms of mg. of adrenal weight per 100 Gm. of body weight, however, no significant difference was noted between the various groups. Adrenal weight in mg. per 100 Gm. of body weight was significantly greater for all promin-fed rats than for animals fed similar rations with promin omitted. In the promin series adrenal weights averaged 37.4 to 39.8 mg. per 100 Gm. of body weight on the various diets employed; in promin-free controls the average range was 24.9 to 28.5 mg. per 100 Gm. of body weight (Table II).

During the eighth week of feeding total and differential white cell counts, hemoglobin determinations, reticulocyte counts and total red cell counts were made on the tail blood of surviving rats on diets A_1, F_1, and A_2. No significant difference in total and differential white cell counts were observed in animals fed any of the above diets. The erythrocyte count and hemoglobin level were somewhat depressed on diet A_1, with erythrocytes averaging 5.8 million (range 4.5 to 6.2) per cubic millimeter of blood and hemoglobin averaging 12.4 Gm. (range 11.4 to 13.7) per 100 cc. of blood. The average erythrocyte count on diet F_1 was 8.1 million (range 7.8 to 8.5) per cubic millimeter of blood, and the average determination of hemoglobin was 15.1 Gm. per 100 cc. of blood (range 14.8 to 15.8). On diet A_2 the average erythrocyte count was 8.0 million (range 7.6 to 8.3) per cubic millimeter of blood, and the average hemoglobin determination was 15.3 Gm. (range 14.5 to 16.3). Reticulocyte counts on the three diets averaged 12.0% on diet A_1 (range 6.9 to 16.0%), 9.9% on diet F_1 (range 4.1 to 17.0%), and 7.0% on diet A_2 (range 4.7 to 11.0%). In previous work Higgins (9) reported a marked reticulocytosis and a significant depression in the erythrocyte count and hemoglobin level of rats fed promin and maintained on a synthetic ration.

[1] Statistically, gain in body weight was significantly greater on diet F_1 than on diet A_1 (see Table II). Furthermore, no overlapping occurred between the two groups, the largest animal on diet A_1 gained less weight than the smallest on diet F_1.

TABLE I.—COMPOSITION OF EXPERIMENTAL DIETS[a]

Components of Basal Mixture	Diets A_1 and A_2	Diets B_1 and B_2	Diets C_1 and C_2	Diets D_1 and D_2	Diet E_1	Diet F_1
Yeast[b]	0.0	0.0	10.0	0.0	0.0	0.0
Whole Liver Powder[c]	0.0	0.0	0.0	10.0	0.0	0.0
Liver Concentrate 1:25[d]	0.0	0.0	0.0	0.0	4.0	0.0
Extracted Liver Residue[e]	0.0	0.0	0.0	0.0	0.0	10.0
Casein[f]	22.0	22.0	22.0	22.0	22.0	22.0
Salt Mixture[g]	4.5	4.5	4.5	4.5	4.5	4.5
Sucrose	73.5	73.5	63.5	63.5	69.5	63.5
Vitamin supplements						
Fat-soluble vitamins: *for all diets*, see footnote [h].						
B vitamins: *for diets* A, C, D, E, and F, see footnote [i]; *for diet* B, see footnote [j].						

[a] Promin (sodium *p,p'*-diaminodiphenylsulfone-*N,N'*-didextrose sulfonate) was incorporated in diets A_1, B_1, C_1, D_1, E_1, and F_1 at a level of 5.0 Gm./Kg. of diet, replacing an equal amount of sucrose.
[b] Brewer's Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.
[c] Whole Dried Liver Powder, Armour and Co., Chicago, Ill.
[d] Liver Extract Concentrate 1:25, Armour and Co., Chicago, Ill.
[e] Liver Residue Powder, Armour and Co., Chicago, Ill.
[f] Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio
[g] Sure's Salt Mixture No. 1 (10).
[h] Each rat received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg., alpha-tocopherol acet 1.5 mg., and a vitamin A–D concentrate containing 50 U. S. P. units of vitamin A and 5 U. S. P. units of vitamin D. T concentrate used was Nopco Fish Oil Concentrate assaying 800,000 U. S. P. units of vitamin A and 80,000 U. S. P. unit vitamin D per Gm.
[i] To each Kg. of diets A, C, D, E, and F were added the following synthetic vitamins: thiamine hydrochloride 72 m riboflavin 9 mg., pyrodoxine hydrochloride 15 mg., calcium pantothenate 67.2 mg., nicotinic acid 60 mg., 2-methyl-1,4-na thoquinone 5 mg. and choline chloride 1.2 Gm. The thiamine, riboflavin, pyridoxine, pantothenate, and nicotinic acid w administered in amounts comparable to that provided by Brewer's Type Yeast No. 200 when fed at a level of 10% of diet. The amounts fed were considerably in excess of minimum requirements for the rat.
[j] To each Kg. of diet B were added thiamine hydrochloride 144 mg., riboflavin 18 mg., pyridoxine hydrochloride 30 calcium pantothenate 134.4 mg., nicotinic acid 120 mg., *p*-aminobenzoic acid 600 mg., folic acid 10 mg., biotin 1 mg., 2-met 1,4-naphthoquinone 10 mg., choline chloride 1.2 Gm., and inositol 1.2 Gm.

TABLE II.—EFFECTS OF PROMIN ADMINISTRATION ON GROWTH AND THYROID, ADRENAL AND OVARI WEIGHT IN THE IMMATURE FEMALE RAT

Dietary Group	Number of Animals	Initial Body Weight, Gm.	Gain in Body Weight Over 8-Week Period, Gm.	Ovarian Weight,[a] Mg.	Adrenal Weight,[a] Mg.	Adrenal Weight, Mg./100 Gm. Body Weight	Thyroid Weight, Mg. Average	Thyroid Weight, Mg. (Range)	Thyroid Weight Mg./10 Gm. Body Weight
				Promin Series					
A_1	10	44.0	51.2 ± 3.0 (5)[b]	24.8 ± 3.3	36.8 ± 1.4	38.7	35.4	(17.2 – 56.0)	37.3
B_1	10	44.0	45.0 ± 3.8 (7)	21.4 ± 1.8	35.4 ± 2.1	39.8	54.0	(40.6 – 88.6)	60.7
C_1	10	44.1	51.5 ± 3.6 (10)	23.0 ± 2.9	35.5 ± 2.9	37.4	32.2	(20.0 – 40.2)	33.5
D_1	10	44.1	67.8 ± 3.1 (8)	30.8 ± 1.8	44.5 ± 3.3	39.7	50.3	(38.1 – 70.4)	44.9
E_1	10	44.3	64.9 ± 2.6 (9)	31.8 ± 2.8	41.2 ± 1.3	37.8	58.0	(32.0 – 98.6)	53.2
F_1	10	44.4	93.9 ± 2.7 (9)	46.7 ± 2.9	51.6 ± 2.2	37.4	62.1	(34.3 – 114.6)	45.0
				Promin-free Controls					
A_2	6	44.5	135.7 ± 5.8 (6)	52.3 ± 3.2	44.8 ± 1.5	24.9	11.9	(10.0 – 15.1)	6.6
B_2	6	44.3	140.0 ± 3.7 (6)	58.4 ± 2.7	49.7 ± 1.5	27.0	10.5	(9.3 – 12.2)	5.7
C_2	6	44.2	133.4 ± 5.5 (6)	55.7 ± 2.0	49.3 ± 2.4	27.7	10.3	(9.1 – 11.0)	5.8
D_2	6	44.0	152.3 ± 5.1 (6)	57.9 ± 2.8	55.8 ± 2.7	28.5	10.7	(8.0 – 14.2)	5.5

[a] Including standard error of the mean calculated as follows: $\sqrt{\frac{\epsilon d^2}{n-1}}/\sqrt{n}$ where d is the deviation from the mean a n is the number of observations.
[b] The values in parentheses indicate the number of animals which survived and on which averages are based.

Under conditions of the present experiment, however, the blood picture of promin-fed rats on the synthetic ration (diet A_1) was virtually normal.

DISCUSSION

It is becoming increasingly apparent that the effects obtained in an experimental animal following administration of drugs or related products are d pendent on the nutritional state and the die employed. In acute deficiencies of essential n trients it is readily recognized that resulting a normalities in cellular metabolism may profound affect response to certain drugs. What is less w recognized, however, is that these drugs themselv

may precipitate a deficiency state. Thyroid administration, for example, may induce a deficiency of thiamine, pyridoxine, and pantothenic acid (11), while dicumarol, salicylates and other drugs may precipitate a deficiency of vitamin K (12–14). In addition to the known nutrients, however, requirements for various unknown factors may also be increased following the administration of certain drugs. These "minor vitamins" are apparently dispensable under normal conditions or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to the extent that deficiencies occur, manifested by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient (15). Whole liver is a potent source of such unknown nutrients. The beneficial effects of liver in animals inhaling carbon tetrachloride or fed toxic amounts of strychnine, sulfanilamide, dinitrophenol, atabrine, and other drugs have been recognized for years (16–18). Similar results have been observed following toxic doses of diethylstilbestrol (16), alpha-estradiol (19) and desiccated thyroid (20–22). In the present experiment, toxic effects of promin, as previously reported by Higgins (9), were similarly modified by dietary means.

Findings indicate that the effects of promin administration in the immature female rat were dependent in part on the diet employed. A marked retardation in growth was observed on all rations. Gain in body weight was greater, however, on diets containing liver or liver fractions (diets D_1, E_1, and F_1) than on the synthetic rations (diets A_1 and B_1) or the ration containing yeast (diet C_1). Growth was particularly marked on diet F_1 (containing the water-insoluble extracted liver residue). On promin-free rations no significant difference on growth was observed on any of the diets employed. Similar findings were obtained in respect to ovarian weight. A marked inhibition of ovarian development was observed in promin-fed rats on synthetic rations and the diet containing yeast. Ovarian weight was somewhat greater in promin-fed rats on diets containing whole liver or water-soluble liver concentrate. Ovarian weights were virtually normal, however, on promin-fed rats receiving the water-insoluble extracted liver residue. On promin-free rations no significant difference in ovarian weight was observed on any of the diets employed. Available data indicate that the beneficial effects of liver and liver fractions on growth and ovarian development in the immature promin-fed rat were not due to any of the known B vitamins. This is indicated by the fact that body and gonadal weight of animals fed diet B_1 was significantly less than that of animals fed diets D_1, E_1 and F_1 although diet B_1 contained all known members of the vitamin B complex[2] in amounts exceeding their content in the latter rations. It is suggested, therefore, that whole liver contains some factor(s) other than the known B vitamins whose requirement is increased following prolonged promin feeding, and that this factor(s) is present primarily in the water-insoluble fraction. This factor is not present in significant amounts in yeast.

No measurements of food consumption were made during the experiment, but it was apparent that promin-fed rats on the synthetic rations (diets A_1 and B_1) ate less food than those receiving the liver supplements. This was particularly marked during the first week of feeding. It is not unlikely that the differences in growth and gonadal development of promin-fed rats on the various rations reflect differences in caloric intake. The paired feeding technique might be employed with advantage in such experiments. On the other hand since promin was mixed in the diet, animals with the largest food consumption were also ingesting the largest doses of promin. An increased caloric intake under these conditions might indicate, therefore, that the diet contained some factor(s) which increased resistance to promin intoxication.

In addition to retardation of growth and inhibition of ovarian development, promin administration under conditions of the present experiment resulted in a marked enlargement of the thyroid gland similar to that caused by promizole (23) and other goitrogenic agents (24). Adrenal weight (expressed in mg. per 100 Gm. of body weight) was also significantly increased in all promin-fed rats. These effects occurred on all promin-containing rations and were not inhibited by any of the diets employed.

SUMMARY

The administration of toxic doses of promin to immature female rats maintained on a synthetic diet resulted in a marked retardation of growth and inhibition of ovarian development. These effects were largely counteracted by the administration of a water-insoluble fraction of liver. The protective factor(s) was distinct from any of the known B vitamins and was not present in significant amounts in yeast. The suggestion is made that whole liver contains at least one factor other than the known B vitamins whose requirement is increased in the promin-fed rat.

Other effects of promin administration included a marked increase in thyroid weight and an increase in the ratio of adrenal to body weight. These effects were not counteracted by administration of the liver factor.

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[2] The recently crystallized vitamin B_{12} is a possible exception to this statement. The latter substance, however, is water-soluble and appears to be distinct from the protective factor in the present experiment which is retained in the water-insoluble fraction of liver.

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Some Pharmaceutical Applications of Statistical Sampling Plans*

By R. H. NOEL†

Specific illustrations of single, double, and sequential sampling plans for various attribute characteristics are presented. Also included is an illustration of the statistical technique employed in the sampling of substances for which the quality characteristics are expressed as a continuous variable. The use of Analysis of Variance in acceptance sampling is illustrated.

SAMPLING inspection for acceptance purposes is carried out at many stages in pharmaceutical manufacturing. The inspection or control analysis is usually performed on a sampling basis because of the destructive nature of much of the testing employed. For this reason, and because of various economic considerations, one hundred per cent testing is precluded.

The pharmaceutical industry has long recognized this problem and as a result it has been responsible for developing rigidly controlled manufacturing systems which are operated by highly trained production personnel. Although these manufacturing controls provide the largest measure of quality assurance, the control laboratories must perform the necessary inspection to insure the desired quality level. Much of this inspection must be performed on a sampling basis whether the testing involves raw materials, packaging materials, intermediate products or finished products. The purpose of this paper is to illustrate some applications of well-known statistical sampling plans to some of the inspection problems of the industry. No attempt will be made to discuss the statistical theory involved as this has been done adequately elsewhere (1–4).

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I. ATTRIBUTE INSPECTION

Consider first, the problems of attribute inspection which arise during the examination of packaging materials, particularly when such material is to be handled in modern, automatic packaging machines. For example, the examination of collapsible tubes for length, diameter, wall thickness, threads, cap diameter, and smears and scratches on lithograph work; closures for size, thread uniformity, pitch and strength of plastic; cap liners for type and resistance; cartons for position of glue flaps, folding and gluing defects, and printing errors constitute some of the many characteristics which require control in order to obtain efficient packaging output.

It is recognized immediately that defects of the kind mentioned above will ordinarily be detected and eliminated at some subsequent inspection station. However, the efficiency of the packaging operation may be considerably impaired if too much defective packaging material is allowed to reach that stage. How much inspection of such material is necessary is, therefore, determined in the light of economy. The problem is one of choosing an inspection plan which will involve a minimum amount of inspection expense, and simultaneously insure that no more than a specific proportion of defects will reach the next stage.

Consider the examination of collapsible tubes for the multiple defects mentioned above. Without one hundred per cent inspection, it would be impossible to insure that every tube will meet all specifications. One hundred per cent inspection, however, would be in all probability far too expensive. Therefore, it is necessary to agree to accept a certain proportion of defective tubes, which proportion shall be determined in the light of the economy of inspection and processing. For example, it may be economically feasible to use lots of tubes where the average proportion defective for all causes is 1.0%. It may be totally uneconomical to utilize lots where the proportion defective is 2.5%. Since one hundred per cent inspection has been adjudged too expensive, it will be necessary to consider the risks involved in accepting bad lots of tubes and in rejecting good lots

of tubes. When values for these two risks have been assigned, it is possible to construct three types of sampling plans: i.e., single, double, and sequential. Assuming that unsatisfactory quality lots should be rejected 95% of the time and satisfactory quality lots should be accepted 95% of the time, it is possible to construct a single sampling plan as follows:

Let

p_1 = acceptable fraction defective = 0.01
p_2 = unacceptable fraction defective = 0.025
α = risk of rejecting a lot of p_1 quality = 0.05
β = risk of accepting a lot of p_2 quality = 0.05

$$R_0 = p_2/p_1 = \text{operating ratio} = \frac{0.025}{0.01} = 2.5$$

Tables for designing sampling schemes have been published by Peach (1) for single and double sampling. They have been reproduced in Tables I and II Find R_0 in Table I. If it does not appear read the next higher number. Read directly the acceptance number c and sample index np_1. Divide np_1 by p_1 to get n, the sample size. From the table, c = 12 and np_1 = 7.69 for R_0 = 2.5 and np_1/p_1 = 769. The sampling scheme, therefore, calls for a sample size of 769 and an acceptance number of 12. That is, if no more than 12 defects are found in a random sample of 769 tubes, the probability is 0.95 that the lot is less than 2.5% defective and the lot is accepted. If more than 12 defects are found, the lot is rejected. If 769 appears to be a rather large sample, it would be desirable to investigate methods of reducing this number. This could be accomplished by increasing the risks, α and β, but the safer and more effective means would be to investigate the economic effect of increasing p_2.

Assuming the same set of conditions, it is possible to design a double sampling plan as follows:

$$p_1 = 0.01$$
$$p_2 = 0.025$$
$$\alpha = 0.05$$
$$\beta = 0.05$$
$$R_0 = p_2/p_1 = \frac{0.025}{0.01} = 2.5$$

From Peach's table for double sampling (Table II), read for R_0 = 2.6 (next largest number from 2.5), c_1 = 5, k_1 = 11, c_2 = 13, and n_1p_1 = 4.34. Calculate $n_1 = n_2 = n_1p_1/p_1$ = 4.34/0.01 = 434. Take a random sample of 434 tubes and inspect them for defects. If no more than 5 tubes are defective, accept the lot. If 11 tubes or more are defective, reject the lot. If more than 5 tubes but less than 11 tubes are defective, take another 434 tubes and inspect them. If no more than 13 tubes are defective in the combined samples, accept the lot. If more than 13 tubes are defective in the combined samples, reject the lot. The lot in question, whether passed on the first or second sample, will have a probability of 0.95 of being less than 2.5% defective. Other considerations being equal, the amount of inspection necessary in double sampling is less than for single sampling and is especially useful where the average percentage defective is low. Still another solution is available which requires, on the average, less sampling. This is known as sequential analysis (2).

Using the same numerical quantities as before, the sequential plan is devised as follows:

$$p_1 = 0.01$$
$$p_2 = 0.025$$
$$\alpha = 0.05$$
$$\beta = 0.05$$
$$g_1 = \log\frac{p_2}{p_1} = \log\frac{0.025}{0.01} = 0.3979$$
$$g_2 = \log\frac{1-p_1}{1-p_2} = \log\frac{0.99}{0.975} = 0.0065$$
$$a = \log\frac{1-\beta}{\alpha} = \log\frac{0.95}{0.05} = 1.2787$$
$$b = \log\frac{1-\alpha}{\beta} = \log\frac{0.95}{0.05} = 1.2787$$
$$h_1 = \frac{b}{g_1+g_2} = \frac{1.2787}{0.3979+0.0065} = 3.16$$
$$h_2 = \frac{a}{g_1+g_2} = \frac{1.2787}{0.3979+0.0065} = 3.16$$
$$s = \frac{g_2}{g_1+g_2} = \frac{0.0065}{0.3979+0.0065} = 0.0161$$

Two parallel lines may now be computed according to the equations: $d_2 = h_2 + sn$ for the upper line and $d_1 = -h_1 + sn$ for the lower line, where n is equal to the number of items being inspected. The lines are plotted on cross-hatched paper as shown in Fig. 1. The vertical axis represents the total number of defectives found and the horizontal axis represents the total number of tubes examined.

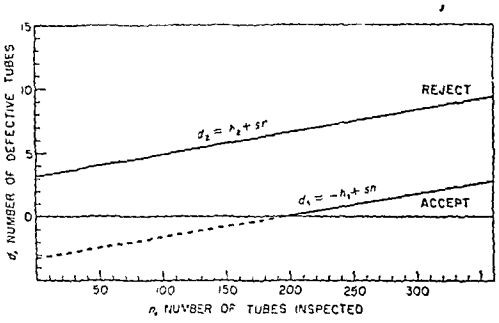

Fig. 1.—Sequential Plan—Graphical Solution Key: p_1=.01; p_2=.025; α=.05; β=.05.

The inspection consists of examining one tube at a time drawn at random from the lot. After each tube is inspected, the inspector plots the total number of defective tubes against the total number of items examined. Each plotted point is thus always one space to the right of the preceding point and it is either on the same level or one space higher depending on whether or not a defective tube is found. Inspection continues until a plotted point falls either into the accept or reject area.

The sequential method permits a decision concerning a lot in accord with the predetermined risks and quality levels with less inspection than single or double sampling, other considerations being equal. It is exceptionally efficient when lots of material are excessively bad or exceptionally good, as little inspection is necessary in such cases either to reject or accept.

The respective values of p_1, p_2, α, and β may differ widely with the material to be inspected, economy involved, and the quality level desired.

II. VARIABLES INSPECTION

Much of the inspection associated with raw materials, in process and finished product control is based upon chemical, physical, and biological analysis. With such types of testing procedures, the quantities being measured are usually capable of being represented on a continuous scale. One hundred per cent inspection is hardly ever possible because of the destructive nature of many of the testing procedures.

A simple example of this type of inspection problem would be the routine laboratory check of the label potency of a finished product. Given a lot of vials with a label claim of 5000 units and minimum and maximum average tolerances of 4000 and 6000 units, respectively, how many vials should be tested in order to be reasonably assured that the potency of the product is satisfactory?

To answer this question, some knowledge of the variability between vials in the same lot must be obtained. This variability is usually accounted for largely by the variability of the filling equipment and the error of the analytical technique, since such vials are filled from homogeneous bulk material. Therefore, the average variability between vials of the same lot is or should be known and well controlled. It should be assumed that the standard deviation is known to be approximately 450 units.

Because of the destructive nature of the testing procedure, the laboratory must resort to sampling inspection and it will, therefore, be necessary to assign values to the two risks associated with the decision to be based on the results of the sample tests. As in attribute sampling, these risks are α, the chances of rejecting a lot when it is of satisfactory potency and β, the chances of accepting a lot when it is of unsatisfactory potency.

The sampling plan can be calculated using Clifford's terminology (5). Let

n = number of individuals in the sample
σ = standard deviation = 450
$\bar{x}$ = average of the sample
$\bar{x}_1$ = lower acceptance average for sample
$\bar{x}_2$ = upper acceptance average for sample
u = computed average of particular lot
u_0 = desired value of lot average = 5000
u_1 = lower tolerance value for lot average = 4000
u_2 = upper tolerance value for lot average = 6000
α = risk of rejecting lot if $u = u_0 = 0.05$
β = risk of accepting lot if $u = u_1$ or $u_2 = 0.05$
$t(\alpha)$ = the value of t on a normal curve corresponding to a one-tail area of α
$t(\beta)$ = the value of t on a normal curve corresponding to a one-tail area of β

Calculate:

$$n = \sigma^2[t(\alpha/2) + t(\beta)]^2/(u_0 - u_1)^2$$
$$n = 450^2[1.960 + 1.645]^2/(5000 - 4000)^2$$
$$n = 2.63$$
$$\bar{x}_1 = t(\alpha/2)u_1 + t(\beta)u_0/[t(\alpha/2) + t(\beta)]$$
$$\bar{x}_1 = [(1.960)(4000) + (1.645)(5000)]/[1.960 + 1.645] = 4456$$

Since $\bar{x}_2$ will be as much above u_0 as $\bar{x}_1$ is below it, we find $\bar{x}_2 = 5544$. From the above calculations, our sampling plan is defined as follows:

1. Analyze 3 vials separately.
2. Calculate the average of the sample $\bar{x}$.
3. If $\bar{x}$ is between 4456 and 5544, accept the lot.
4. If $\bar{x}$ is less than 4456 or over 5544, reject.

III. APPLICATION OF VARIANCE ANALYSIS TO A CHEMICAL SAMPLING PROBLEM

It is not uncommon for the purchasing agent or the production manager to request an estimate of the active constituent in a large shipment of raw material. The purchasing agent is interested in learning whether he has purchased what he has specified and, moreover, he is interested in a rapid answer in order that he may take advantage of discounts on cash payments. The production manager may be interested from many standpoints, not the least of which is that he must be reasonably sure that sufficient active constituent is available to produce a specific amount of finished product over a period of time.

Assuming that adequate analytical methods are available for the precise determinations of the active constituent involved, one must concentrate upon obtaining a sample which is representative of the shipment. That the content of the active constituent in raw materials may vary from time to time, is unquestioned. What is more disconcerting is the fact that the variability between shipments, lots, barrels, bags, etc., may be such that no standard sampling technique will serve for every instance. It, therefore, becomes important to sample in such a manner that a measure of all possible variation will be obtained. What is more important is to be able to separate the total variability inherent in the shipment into the component parts which are associated with each factor such as lots, barrels, bags, etc. Once this information is available, it is quite possible to define the exact method of taking one additional sample which will supply all the information necessary to make a precise estimate of the quality of the shipment.

The answer to some of these problems can be found by application of a statistical technique known as "Variance Analysis," the fundamentals of which are described in many standard texts (6–8). The following example will serve to illustrate such an application:

One consignment of concentrated aluminum hydroxide gel was received in two lots of 50 barrels each. The consignment was alleged to be homogeneous. The label claim was 8% plus or minus 1% Al_2O_3.

The production department wished to know if the true mean value of the entire consignment was between 7% and 9% Al_2O_3 so that they could determine whether or not they would have enough material to process a desired amount of product containing 4% Al_2O_3. Further, it was the responsibility of the control department to accept or reject the material on the basis of 8% plus or minus 1% Al_2O_3 as per purchasing specifications.

The nature of this material was such that at first glance it would seem that every barrel would require

an analysis, for water had separated and some settling had occurred. For the purpose of acceptance sampling, however, such detailed sampling was not necessary, as will be shown below.

It was decided to take two samples from each of two barrels from each lot and perform duplicate analyses. It is obvious that the purpose of this sampling was to ascertain the variability between lots, between barrels, within lots, between samples within barrels as compared with the variability in the analytical technique.

The results are tabulated below:

Lot 1, % Al_2O_3

Assay	Barrel 1 Sample 1	Barrel 1 Sample 2	Barrel 2 Sample 1	Barrel 2 Sample 2
1	8.2	8.9	7.2	9.1
2	8.1	9.0	7.3	9.0

Lot 2, %

Assay	Barrel 1 Sample 1	Barrel 1 Sample 2	Barrel 2 Sample 1	Barrel 2 Sample 2
1	10.0	8.9	10.0	9.0
2	10.1	9.2	10.3	9.2

Analysis

1. Square each individual value, and sum these squares, i.e.,

$$(8.2)^2 + (8.1)^2 \ldots + (9.2)^2 = 1299.59$$

2. Square each sample block total, sum these squares and divide by the number of individuals making up each total, i.e.,

$$\frac{(8.2 + 8.1)^2 + \ldots + (9.0 + 9.2)^2}{2} = 1299.46$$

3. Square each barrel block total, sum these squares and divide by the number of individuals making up each total, i.e.,

$$\frac{(8.2 + 8.1 + 8.9 + 9.0)^2 + \ldots + (10.0 + 10.3 + 9.0 + 9.2)^2}{4} = 1293.47$$

4. Square each lot block total, sum these squares and divide by the number of individuals making up each total, i.e.,

$$\frac{(8.2 + 8.1 + \ldots + 9.0)^2 + (10.0 + 10.1 + \ldots + 9.2)^2}{8} = 1293.14$$

5. Obtain the grand total of all observations, square this total and divide by the total number of individual items, i.e.,

$$\frac{(8.2 + 8.1 + \ldots + 9.2)^2}{16} = 1287.02$$

The Variance Analysis Table is set up as indicated in Table III.

The sums of squares for the Variance Analysis Table are obtained as is outlined at the bottom of this page.

It is of first importance to learn whether the between samples variability is sufficiently large to be detected by the analytical method employed. In this case, the mean square value for the samples is 1.4975 and that for the duplicate analyses is 0.01625. It is easily seen that the between sample means square is 92.15 times as large as the between duplicate analysis mean square (1.4975/0.01625). It now becomes a matter of determining if the magnitude of this ratio (92.15) can be considered statistically significant. This ratio is known as the F ratio and probability tables for determining the significance of F are available (7). It was determined from such a table (4 and 8 degrees of freedom) that the analytical method would only fail 5 times in 100 if the ratio were 3.84 and only one time in 100 if the ratio were 7.01 to detect the large variation associated with samples from the same barrel. Since the calculated value far exceeds either tabular value, it is concluded that the variability between samples within a barrel is real and can be detected.

If the variation between barrels is not as great as that within barrels, one cannot detect such a variation from a multiple analysis of the barrel contents. Attention is now focused on the between barrel mean square. The ratio of this mean square to the mean square for samples is obtained: 0.165/1.4975 = 0.110. The tabular value for F at p = 0.05 with 2 and 4 degrees of freedom is 6.94. Inasmuch as the calculated ratio is far less than the tabular value, it should be concluded that variability between barrels is not significant and, therefore, does not exist.

To determine whether a significant variation exists between lots, the mean square for lots is tested against the pooled mean square for samples and barrels, viz.:

$$0.33 + 5.99 = 6.32$$
$$6.32/(2 + 4) = 1.0533$$
$$F = 6.12/1.0533 = 5.81$$

From the F table, the value 5.99 is found for 1 and 6 degrees of freedom at P = 0.05. Since the calculated value is so close to the tabular value, it is suspected that the variation between lots is real and can be detected.

From the equations listed in the column headed "Components of Variance," the following are calculated:

$$(s_4)^2 = 0.0162$$
$$(s_3)^2 = \frac{1.49750 - 0.0162}{2} = 0.7406$$
$$(s_2)^2 = 0 \text{ as determined above}$$
$$(s_1)^2 = \frac{6.12 - 1.4975}{8} = 0.5778$$

The total variance of a single analysis can be calculated as: $(s_1)^2 + (s_2)^2 + (s_3)^2 + (s_4)^2 = 0.5778 + 0.0 + 0.7406 + 0.0162 = 1.3346$.

The standard deviation is the square root of the total variance: $s = \sqrt{1.3346} = 1.16\%$.

Between lots	(4) − (5)	= 1293.14 − 1287.02	=	6.12
Between barrels within lots	(3) − (4)	= 1293.47 − 1293.14	=	0.33
Between samples within barrels	(2) − (3)	= 1299.46 − 1293.47	=	5.99
Between duplicate analyses	(1) − (2)	= 1299.59 − 1299.46	=	0.13
Total sum of squares	(1) − (5)	= 1299.59 − 1287.02	=	12.57

Thus for a single analysis on one sample from one barrel from one lot, the estimate of the Al_2O_3 content with an adequate degree of certainty would only be within $\pm 1.96 \times 1.16 = 2.27\%$ of the true value (6, 7). It is obvious that some other sampling technique must be employed since the estimate must be within $\pm 1\%$ of the true value.

Concentrating attention on the factors contributing the greatest variance, a sampling procedure can be devised to reduce this error of estimate to the required level.

Since (s_2) does not exist, the number of barrels to be sampled is not important because the analysis has indicated that the barrels are similar even though their contents are not homogeneously mixed.

Increasing the number of samples from any one barrel would decrease the error of estimate only as affected by within-barrel heterogeneity.

Increasing the number of barrels sampled from each lot would decrease the error of estimate only as affected by lot variability.

The conclusion is that there is a difference in the Al_2O_3 content of the two lots. The lots are in themselves homogeneous; the variation within barrels of the same lot arises from the settling which occurred on standing. Therefore, either 1 sample from each of 10 barrels in each lot or 10 samples from 1 barrel in each lot will equally increase the precision of the estimate of the lot difference. Using the latter scheme, however, will simultaneously provide an increase in the precision of the estimate of within barrel differences. The number of analyses would not necessarily increase because blending of the samples has the effect of adding the variances into a homogeneous sample.

Therefore, it was decided to observe the effect of taking 10 samples with a sampling thief from 1 barrel from each lot, and blending all samples. The estimated variance of an analysis performed on the blended sample would be:

$$\frac{0.5778 + 0.7406}{10} + 0.0162 = 0.1480$$

and the standard deviation:

$$s = \sqrt{0.1480} = 0.385\%$$

The estimated Al_2O_3 content would now be within $\pm 1.96 \times 0.385 = 0.755\%$ which is adequate for the problem at hand. Thus, with one more sample taken as described above and with but a single additional analysis, the Al_2O_3 content of the shipment can be estimated well within the desired range of $\pm 1\%$ Al_2O_3.

SUMMARY

The use of some statistical sampling methods in pharmaceutical inspection problems has been

TABLE I.—FOR DESIGNING SINGLE SAMPLING SCHEMES ($\alpha = \beta = 0.05$)[a]

R_0	c	np_1
58.	0	0.051
13.	1	0.355
7.5	2	0.818
5.7	3	1.366
4.6	4	1.970
4.0	5	2.61
3.6	6	3.29
3.3	7	3.98
3.1	8	4.70
2.9	9	5.43
2.7	10	6.17
2.5	12	7.69
2.37	14	9.25
2.03	21	14.89
1.81	30	22.44
1.61	47	37.20
1.51	63	51.43
1.335	129	111.83
1.251	215	192.41

[a] This table was reproduced from Paul Peach's "Industrial Statistics and Quality Control" (1) by permission of the author.

TABLE II.—FOR DESIGNING DOUBLE SAMPLING PLANS ($\alpha = \beta = 0.05$)[a]

R_0	c_1	k_1	c_2	n_1p_1
15.1	0	1	1	0.207
8.3	0	2	2	0.427
5.1	1	3	4	1.00
4.1	2	4	6	1.63
3.5	2	5	7	1.99
3.0	3	7	9	2.77
2.6	5	11	13	4.34
2.3	6	13	16	5.51
2.02	9	17	23	8.38
1.82	13	23	32	12.19
1.61	21	34	50	20.04
1.505	30	45	69	28.53
1.336	63	83	138	60.31

[a] This table was reproduced from Paul Peach's "Industrial Statistics and Quality Control" (1) by permission of the author.

TABLE III

Source of Variation	Degrees Freedom[a]	Sum of Squares	Mean Square	Components of Variance
Between lots	1	6.12	6.12	$n_2n_3n_4(s_1)^2 + n_3n_4(s_2)^2 + n_4(s_3)^2 + (s_4)^2$
Between barrels within lots	2	0.33	0.165	$n_3n_4(s_2)^2 + n_4(s_3)^2 + (s_4)^2$
Between samples within barrels	4	5.99	1.4975	$n_4(s_3)^2 + (s_4)^2$
Between analyses on same sample	8	0.13	0.0162	$(s_4)^2$
Total	15	12.57		

[a] The degrees of freedom are obtained as follows: Let n_1 = number of lots = 2; n_2 = number of barrels from each lot = 2; n_3 = number of samples from each barrel = 2; and n_4 = number of analyses on each sample = 2.

Then the degrees of freedom for: Lots = $(n_1 - 1) = (2 - 1) = 1$; barrels = $n_1(n_2 - 1) = 2(2 - 1) = 2$; samples = $n_1n_2(n_3 - 1) = (2)(2)(2 - 1) = 4$; analyses = $n_1n_2n_3(n_4 - 1) = (2)(2)(2)(2 - 1) = 8$; total = $(n_1n_2n_3n_4) - 1 = (2)(2)(2)(2) - 1 = 15$.

illustrated. Single, double, and sequential sampling methods were applied to the attribute inspection of packaging material. A sampling method for variables inspection of a finished product is included along with an illustration of the use of variance analysis in chemical sampling.

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Studies in the Hydrophilic Properties of Possible Ointment Base Constituents. II. The *n*-Alkyl Alcohols with a Carbon Chain Length of C_{20} to C_{30}*

By ALFRED HALPERN† and WILLIAM J. WILKINS‡

The *n*-alkyl alcohols from C_{20} to C_{30} were synthesized and the physical constants of the alcohols and their intermediates were determined. The water numbers of these alcohols were determined and the optimum concentration of the alcohol causing the maximum water potentiation was noted. The C_{20} alcohol caused the greatest water potentiation and the C_{24} alcohol had the lowest optimum concentration. The alcohols with the carbon chain length greater than 24 had the same optimum concentration, 3 per cent. The study indicates that molecular size and the orientation of the alkyl group (lipophilic) plays an important part in the emulsifying properties of the fatty alkyl alcohols.

WATER numbers of the fatty alcohols with a carbon chain length of C_{10} to C_{18} have been reported (1). The optimum concentration of the fatty alcohols necessary to cause the maximum potentiation of the water absorbing properties of petrolatum increased with the increase in carbon chain length. Cetyl alcohol, however, had the lowest optimum concentration (4%) while stearyl alcohol caused the greatest water absorbtion with 43.2 cc. of water per 100 Gm. of fatty alcohol-petrolatum mixture. Rupp (2) also reported better water absorbing properties of fatty alcohols, with increased carbon chain length.

Since the amount of water absorbed by the fatty alcohol-petrolatum mixtures previously reported (1) continued to increase with the carbon chain length it was of interest to determine the optimum carbon chain length that caused the maximum water absorption of petrolatum. The present study concerns itself with the *n*-alkyl primary alcohols with a carbon chain length from C_{20} to C_{30}.

The higher *n*-alkyl alcohols are present as constituents of natural waxes (3). The isolation of alcohols from these sources was not a convenient procedure. There are many synthetic methods described in the literature for the preparation of these alcohols. They may be prepared by the reduction of the appropriate fatty acid ester. Generally these are prepared by the successive increments of one or two carbon atoms starting from stearic acid (4). Robinson (5) reported a method whereby long chain acids were obtained through one operation. The alcohols could be obtained from the acid through the Bouveault-Blanc reduction of the ester.

Recently, Jones (6) reported a method for the preparation of long chain primary alcohols through the reaction between alkyl zinc compounds with ω-acetoxy acyl halides. This method eliminates the necessity of a Bouveault-Blanc reduction.

$$\underset{(I)}{RZnX} + \underset{(II)}{XCO(CH_2)_nCH_2OOCCH_3} \longrightarrow \underset{(III)}{R{-}CO{-}(CH_2)_nCH_2OOCH_3}$$

$$\text{III} \xrightarrow[HCl]{Zn\cdot Hg} \underset{(IV)}{RCH_2 - (CH_2)_n - CH_2OOCCH_3}$$

$$\text{IV} \xrightarrow{NaOH} \underset{(V)}{RCH_2 - (CH_2)_n - CH_2OH}$$

A limitation of this method was the difficulty in obtaining the hydroxy acids, since these acids

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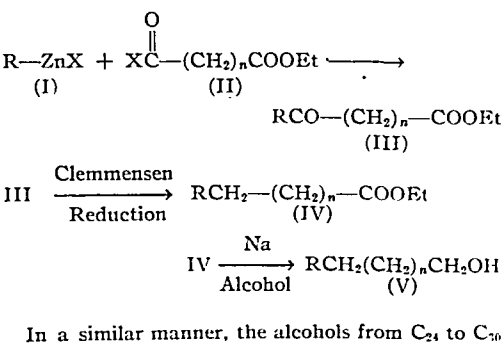
were obtained from the decomposition of the appropriate ozonide (13).

The alcohols used in this study were prepared through a modification of a method described by Jones (6). Another preparative procedure was employed to obtain eicosyl alcohol.

EXPERIMENTAL

Preparation of Eicosyl Alcohol ($C_{20}H_{41}OH$).—The Grignard reagent was prepared in the usual manner from 0.1 mole (30.4 Gm.) of stearyl chloride and 0.12 mole (2.5 Gm.) of magnesium and 500 cc of anhydrous ether. When all of the stearyl chloride was added the mixture was stirred for 1 hr. and then a solution of 0.2 mole of ethylene oxide in 300 cc. of anhydrous ether was added dropwise as fast as the condenser would allow. The reaction was very violent. The temperature of the reaction mixture was kept at 0° by means of an ice-salt bath. When the addition was completed the mixture was allowed to warm at room temperature and then worked up in the usual manner. The ether layer was separated, dried over anhydrous sodium sulfate and then the solvent was distilled. The residue was crystallized from benzene, m. p. 69–70°. Yield: 71% theoretical.

An attempt was made at the preparation of docosyl-alcohol in a manner similar to that described above. However, the yield of cosyl bromide prepared by the method described (7) for the preparation of alkyl bromides was 18%. Since a moderately large quantity of the alcohol was needed, this method (i.e., through the Grignard) of obtaining the alcohol was not satisfactory. The alcohol was prepared in a fairly good yield, by a method described by Jones (6) employing ethyl succinyl chloride and stearyl zinc chloride. The resulting long chain ketonic acid ester was reduced with amalgamated zinc through the Clemmensen method, to ethyl docosanate. The resulting long chain aliphatic ester was reduced to docosanol through a Bouveault-Blanc reduction.



In a similar manner, the alcohols from C_{24} to C_{30} were prepared.

Preparation of Monoethyl Esters of Dicarboxylic Acids.—The monoethyl esters of the dicarboxy aliphatic acid were prepared through the method described in "Organic Syntheses" (8) for the preparation of ethyl hydrogen sebacate (Table I).

These acid esters were then converted to the acyl chloride by refluxing the appropriate compound with 0.2 *M* excess of thionyl chloride, for four hours. The excess thionyl chloride was removed by distillation under diminished pressure and the acyl chloride distilled at 1–2 mm. pressure.

Preparation of Ketonic Acids.—Stearyl bromide[1] was prepared from Stearyl alcohol[2] in the manner

TABLE I.—THE MONOETHYL ESTERS OF THE DICARBOXYLIC ACIDS—($C_2H_5OOC(CH_2)_nCOOH$)

n =	Formula	B. P., 1–2 Mm	Yield, %
2	$C_6H_{10}O_4$	139–140	87
4	$C_8H_{14}O_4$	149–150	84
6	$C_{10}H_{18}O_4$	153–154	76
8	$C_{12}H_{22}O_4$	158–160	71
10	$C_{14}H_{26}O_4$	180–181	72

that was described for the preparation of dodecyl bromide (9).

A solution of 0.2 *M* Grignard reagent prepared in the usual way from stearyl bromide, magnesium, and ether, was added to 0.2 mole of zinc chloride (fused and powdered) and 250 cc. of ether, in a 3-necked flask (fitted with a stirrer, reflux condenser and dropping funnel). When all of the Grignard reagent had been added, the mixture was refluxed for two hours and then the ether distilled until the volume was reduced to one half. A solution of 0.2 mole of the appropriate acyl chloride in 200 cc. of benzene was added. The mixture was refluxed for three hours and then hydrolyzed with dilute acid. The layers were separated and the aqueous layer extracted with benzene. The extracts were combined with the nonaqueous layer and the whole washed with acidulated water. The benzene was removed by distillation and the residue hydrolyzed with 250 cc. of 20% NaOH. The acids were regenerated from the sodium salt by acidification with dilute HCl. The crude acid was collected at the pump and air dried. The acids were purified by repeated reprecipitation from alkaline solution (Table II).

The ketonic acids were reduced to the long chain aliphatic acid through a Clemmensen method. The directions given by R. G. Jones (6) for the reduction of 13-ketohentriacontanoic acid and the esterification of the resultant acid to ethyl hentriacontanoate were followed exactly in order to prepare the ethyl esters of the aliphatic acids from C_{22} to C_{30} (see Table III).

A mixed melting point between the docosyl alcohol and an authentic sample[2] showed no depression. Similarly there was no depression of the melting point of an authentic sample of hexacosyl alcohol (ceryl alcohol, E. K.) mixed with the one prepared above.

Melissyl alcohol was reported (10) to be present as the palmitate in beeswax. An attempt was made to isolate this alcohol from this source.

Isolation of "Triacontanol" from Beeswax.—One hundred grams of beeswax (U. S. P.) were refluxed with 300 cc. of 50% alcoholic KOH for eight hours.

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[1] A sample of Stearyl Bromide was furnished by the Columbia Chemicals Co., Columbia, S. C., through the courtesy of Mr. Max Gergel.

[2] Furnished by the E. I. Du Pont de Nemours Co., Wilmington, Del., through the courtesy of Dr. J. H. Shipp.

TABLE II.—THE KETONIC ACIDS —CH_3—$(CH_2)_{16}$—CH_2—CO—$(CH_2)_n$COOH

n =	Formula	M. P.°	Yield, %	Neutralization Equivalent[a] Calculated	Found
2	$C_{22}H_{42}O_3$	91–93	78	352.6	350.4
4	$C_{24}H_{46}O_3$	94–96	76	392.3	390.4
6	$C_{26}H_{50}O_3$	97–98	74	410.6	410.2
8	$C_{28}H_{54}O_3$	99–100	68	438.7	437.8
10	$C_{30}H_{58}O_3$	101–102	73	466.7	466.3

[a] Shiner, R. L., and Fusion, R. C., "Systematic Identification of Organic Compounds," J. Wiley and Sons, New York, N. Y., ed. 2, p. 116.

TABLE III.—ETHYL ESTERS OF n-ALKYL ACIDS—(CH_3—$(CH_2)_n$COOEt)

n =	Formula	M. P. °	Yield, %
20	$C_{24}H_{48}O_3$	45–46	78
22	$C_{26}H_{52}O_3$	49–50	76
24	$C_{28}H_{56}O_3$	53–54	72
26	$C_{30}H_{60}O_3$	58–59	74
28	$C_{32}H_{64}O_3$	63–64	75

The mixture was poured into 2 L. of cracked ice and the flocculent precipitate collected on a Buchner funnel. The precipitate was washed by repeated suspension in 1 L. of warm water, until the supernatant liquid was neutral to litmus (7 washings). The precipitate was collected and dried. The melting point of the precipitated material indicated a mixture of compounds (m. p. 76–90°). Yield: 48 Gm.

A small portion of this precipitate was sublimed at 1–2 mm. pressure. The sublimate was removed periodically. The first portion melted at 76–82°; sublimation point 150–170°; second portion subliming at 185–200°; melted at 84–88°. The fraction subliming at 200–225° melted at 86–94°.

A larger sample (25 Gm.) was sublimed at 185–200° and 1–2 mm. pressure, the middle fraction collected. M. p. 82–86° (17 Gm.).

The melissyl alcohol ($C_{30}H_{61}OH$) prepared synthetically depressed the melting point of the presumed melissyl alcohol isolated from the beeswax. It was probably a mixture of the C_{28} to the C_{32} alcohols. This was in accord with the report (3) that the alcohols isolated from the natural sources and referred to in the literature as ceryl alcohol ($C_{26}H_{53}OH$) and melissyl alcohol ($C_{30}H_{61}OH$) are invariably mixtures of the even numbered homologs. (See Table IV.)

The water numbers of the alcohols were determined in the manner described by Halpern and Zopf (1). To check these values the amount of water in 2 Gm. of the saturated base was then determined by the U. S. P. XIII toluene distillation method (Table V).

The optimum concentrations of the alcohols and the amount of potentiation of hydrophilic properties of petrolatum were correlated to the carbon chain length (Figs. 1 and 2). The values for the series from C_{10} to C_{18} were obtained from the previously reported study (1) by Halpern and Zopf in order to complete the series.

DISCUSSION

The results of the study indicated that the maximum increase of the water number of petrolatum was caused by the C_{20} alcohol. The optimum concentrations of the n-alkyl alcohols decreased with the increase in the carbon chain length. The optimum as reported for cetyl alcohol by Halpern and Zopf (1) indicates that some factor in addition to the chain length or molecular size was a determinant of the optimum concentration of the alcohols. It is important to note that the cetyl alcohol used in their study was labeled cosmetic

TABLE IV.—THE n-ALKYL ALCOHOLS—$CH_3(CH_2)_nCH_2OH$

n =	Formula	Chemical Name	Common Name	Melting Point Recorded	Found
18	$C_{20}H_{41}OH$	Eicosanol	...	71	69–70
20	$C_{22}H_{45}OH$	Docosanol	...	...	71–72
22	$C_{24}H_{49}OH$	Tetracosanol	...	...	74–76
24	$C_{26}H_{53}OH$	Hexacosanol	Ceryl Alcohol	79.5	77–78
26	$C_{28}H_{57}OH$	Octacosanol	...	83.4	82–83
28	$C_{30}H_{61}OH$	Triacontanol	Melissyl Alcohol	86.5	85–86

TABLE V.—WATER NUMBERS[a] OF ALCOHOLS $C_nH(2_{n+1})OH$

n =	Per Cent Concentration in White Petrolatum[b] 1	2	3	4	5	6	7	8	9	10
20	30.3	38.1	43.1	51.2	54.3	48.3	41.0	38.2	36.0	30.0
22	25.1	31.3	40.1	46.3	43.0	38.0	35.0	32.0	30.0	...
24	24.4	30.0	38.0	35.0	34.0	30.4	28.2	27.1	26.4	...
26	23.0	28.0	36.0	35.6	33.0	28.0	26.1	24.3	22.0	20.0
28	26.0	33.0	35.0	34.1	32.4	30.1	28.0	26.0	24.0	23.0
30	21.0	27.0	33.6	33.1	31.6	29.2	27.1	25.0	24.1	20.8

[a] Based on 100 Gm. of the mixture.
[b] The water number of the petrolatum used was 9.5.

grade and it was not known whether it was derived from natural or synthetic sources. The possibility of impurities should not be discounted.

It is difficult to provide an explanation for the apparent nonconformance of cetyl alcohol. Further work is now in progress to ascertain the manner through which possible impurities influence the optimum concentrations of the alcohol and its water number. Casparis and Meyer (11) also reported the optimum concentration of cetyl alcohol to be 4%.

The leveling off of the optimum concentrations with the C_{24} alcohol indicated the possibility of an optimum molecular size for orientation at the water-oil interface. A study of the relationship between the geometric orientation of the fatty alkyl group and emulsifier efficiency has been started and will be reported at a later date.

The amount of water that could be emulsified in petrolatum compared to the carbon chain length had its maxima at the C_{20} alcohol. It is important to note that while there was a decrease in the amount of water that was emulsified in the petrolatum by the alcohols greater than eicosanol, the series with the chain length from C_{20} to C_{30} caused a greater emulsification than the corresponding series from C_{10} to C_{20}. It was shown that hydrogen bonding or association was a factor in explaining the activity of fatty alcohols as emulsifying agents (12). Assuming this property of the hydroxyl group to be a constant for the series studied, then any differences noted would be due to the change in the lipophilic group. Since this change consisted of an increase in molecular size, a large measure of the properties noted of this series of alcohols presumably were determined by the geometric orientation of the lipophile about the water droplet. The *n*-alkyl alcohols greater than C_{10} are all miscible with petrolatum (within the concentration limits of this study), thus the solubility of the alkyl group in the fat phase ceases to become a deciding factor, again pointing to the spacial configuration of the chain as a determinant.

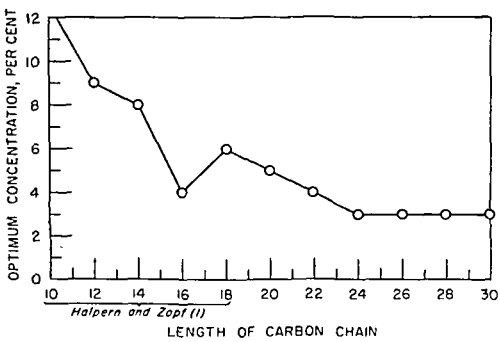

Fig. 1.

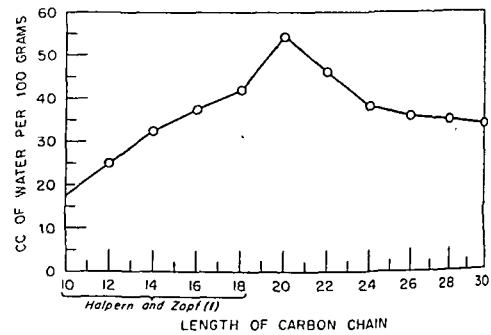

Fig. 2.

SUMMARY

1. The *n*-alkyl alcohols from C_{20} to C_{30} were synthesized and their physical constants and intermediates were determined.

2. The water numbers of these alcohols were determined and the optimum concentration of the alcohol causing the maximum water potentiation was noted.

3. The C_{20} alcohol caused the greatest water potentiation and the C_{24} alcohol had the lowest optimum concentration. The alcohols with the carbon chain length greater than 24 had the same optimum concentration, 3 per cent.

4. The study indicates that molecular size and spacial orientation of the alkyl group (lipophilic) plays an important part in the emulsifying properties of the fatty alkyl alcohols.

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Studies in the Hydrophilic Properties of Possible Ointment Base Constituents. III. The Fatty Mercaptans*

By ALFRED HALPERN† and ARTHUR C. GLASSER‡

The mercaptans with a carbon chain length from C_{10} to C_{18} were synthesized. The physical properties of these compounds and their intermediates were noted. The water numbers of the mercaptans were determined and a correlation was made between the mercaptan series and their hydroxy-analogs. The mercaptan series as a whole showed little ability to emulsify water in petrolatum. The range in water numbers for the mercaptan series was from 15 to 18 at the optimum concentration. The optimum concentration of the mercaptans fluctuated slightly (from 2 to 4 per cent).

THE CHEMISTRY of the mercaptans has been extensively reviewed by Malisoff, Marks, and Hess (1). One outstanding difference between the mercaptan group and the hydroxyl group is the rather limited tendency of the sulfhydryl grouping to undergo association. This has been reported as a result of spectroscopic studies of thiophenol (2) and from heats of mixing studies (3) which showed that the weak donor ability of sulfur was responsible for the failure of thiophenol to be associated. It was also shown (3) that the hydrogen of *n*-heptyl mercaptan did not form bonds with donor oxygen or nitrogen.

The greater association of the lower alcohols causes these compounds to have a higher boiling point than their corresponding sulfur derivatives. In the case of the *n*-heptyl alcohol, its association is enough to compensate for the increased molecular weight of the *n*-heptyl mercaptan so that the boiling point of both these compounds is the same.

As a phase of a study of the mechanisms of hydrophilic ointment formation, it was deemed important to investigate the role of association of the fatty alcohols in these bases. A determination of the water numbers of a homologous series of mercaptans when compared with their hydroxy analogs should give such information. The water numbers of a series of fatty alcohols from C_{10} to C_{18} have been reported (4) and the present study was concerned with the water numbers of a corresponding series of alkyl mercaptans.

Mercaptans have been prepared by the hydrolysis of S-alkylisothiouronium salts (5); the reaction between alkyl halides and metal hydrosulfides (6); hydrolysis of thioesters (7); the addition of hydrogen sulfide to olefins (8); catalytic alkylation of hydrogen sulfide (9); and the acid and alkali reduction of disulfides (10).

The mercaptans[1] used in this study were prepared through the reaction between the alkyl bromide and sodium hydrosulfide in absolute ethanol (I).

$$RX + NaHS \rightarrow RSH + NaBr \qquad (I)$$

The formation of the di-alkyl sulfide was observed to some extent. Apparently this occurred as a result of the sodium mercaptide forming through the reaction between the sodium hydrosulfide and the mercaptan (II).

$$RSH + NaHS \rightleftharpoons RSNa + H_2S \qquad (II)$$

The sodium mercaptide then reacted with another molecule of the *n*-alkyl bromide to form the dialkyl sulfide (III).

$$RSNa + RX \rightarrow RSR + NaX \qquad (III)$$

By increasing the concentration of hydrogen sulfide during the preparation of the sodium hydrosulfide, *in situ*, the formation of the dialkyl sulfide was markedly reduced from 12–19% to 4–6%.

The mercaptans were also prepared through the alkaline hydrolysis of the appropriate S-alkylisothiouronium salt. Dialkylsulfide formation resulted to some extent with this method, too.

The mercaptans were rather easily oxidized by exposure to air and also had a tendency to split out hydrogen sulfide. The mercaptans were converted to the lead mercaptides. The mer-

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[1] The authors gratefully acknowledge the cooperation of Mr. Max Gergel, of Columbia Organic Chemicals Co., Columbia 99, S. C., by furnishing samples of decyl, dodecyl, and tetradecyl mercaptans. These products, described as being "95% pure," were purified by distillation at reduced pressure and were used as reference compounds.

captides may be stored for a reasonable period without the above changes occurring. The mercaptan may be regenerated from the lead mercaptide by warm hydrochloric acid treatment.

The water numbers (4) of the mercaptans were determined and compared with the hydroxy analogs.

EXPERIMENTAL

Preparation of the *n*-Alkyl Bromides.—The alkyl bromides with a carbon chain length from C_{10} to C_{18} were prepared in the manner described by Kamm and Marvel (11) for dodecyl bromide. To increase the yield of the hexadecyl bromide and the octadecyl bromide, the refluxing was stopped at the end of three hours and the layers separated. The acid layer was discarded and a fresh portion of 48% hydrobromic acid was added and the refluxing continued for an additional two hours. The yield of the various alkyl bromides obtained are given in Table I.

TABLE I.—THE ALKYL BROMIDES

Compound	B. P., 12–15 Mm.	Yield, %
$C_{10}H_{21}Br$	123–125	82
$C_{12}H_{25}Br$	148–150	70
$C_{14}H_{29}Br$	170–172	68
$C_{16}H_{33}Br$	193–195	63
$C_{18}H_{37}Br$	214–218	52

Preparation of the *n*-Alkyl Mercaptans.—In a liter flask, fitted with a reflux condenser, was placed 300 cc. of absolute ethanol. One-tenth mole of sodium (2.3 Gm.) was added and when solution was effected, hydrogen sulfide was passed through the reaction medium. When the mixture showed a gain in weight of 10 Gm., 0.1 mole of the appropriate alkyl bromide was added. The mixture was heated to reflux and the stream of hydrogen sulfide was continued through the reaction medium. It was then refluxed for six hours, cooled and filtered, and concentrated to one-third its original volume. The mercaptan separated on dilution with 150 cc. of ice water and was removed with the aid of a separatory funnel or by filtration (stearyl mercaptan). The mercaptans were purified by distillation at reduced pressure (Table II).

TABLE II.—*n*-ALKYL MERCAPTANS

Compound	B. P.,° 1–2 Mm.	% Yield[a]	% Yield[b]	2–4 Dinitrophenylthioether,[c] M. P., °
$C_{10}H_{21}SH$	108	61	66	89
$C_{12}H_{25}SH$	126	72	68	92
$C_{14}H_{29}SH$	149	68	71	93–94
$C_{16}H_{33}SH$	170[d]	70	64	95–96
$C_{18}H_{37}SH$	188[e]	66	58	97

[a] Sodium hydrosulfide method.
[b] S-Alkylisothiouronium Bromide method.
[c] Bost, Turner, & Norton, *J. Am. Chem. Soc.*, 54, 1985 (1932).
[d] M. p. 17–19°.
[e] M. p. 24–26°.

The mercaptans were also prepared by reacting thiourea with the alkyl bromide in the manner described for the preparation of S-methyl-isothiourea sulfate (12). Absolute alcohol was used as the solvent and the period of reflux was lengthened to three hours. The melting points and yields are given in Table III.

TABLE III.—*n*-ALKYL-ISOTHIOURONIUM BROMIDES

n-Alkyl	M. P., °	Yield, %
$C_{10}H_{21}$	99	88
$C_{12}H_{25}$	103	86
$C_{14}H_{29}$	106	83
$C_{16}H_{33}$	110	84
$C_{18}H_{37}$	112	80

The mercaptan was liberated from the isothiouronium bromide by boiling with 5% sodium hydroxide in equimolar proportions. The product was extracted with ether, dried, and purified by distillation at reduced pressure (Table II). Approximately 10 to 18% of the dialkyl sulfide resulted from this method (Table IV).

TABLE IV.—THE DIALKYL SULFIDES

Empirical Formula	Melting Point Found	Melting Point Received[a]	% Yield[b]	% Yield[c]
$(C_{10}H_{21})_2S$	24–25[d]	....	5	10
$(C_{12}H_{25})_2S$	33–34	33.5–34	4	14
$(C_{14}H_{29})_2S$	44–45	45.5–46	6	15
$(C_{16}H_{33})_2S$	51–52	53.5–54	6	12
$(C_{18}H_{37})_2S$	61–62	62.0–62.5	6	18

[a] Fore, D., and Bost, R. W., *J. Am. Chem. Soc.*, 59, 2557 (1937).
[b] Sodium hydrosulfide method.
[c] S-Alkyl isothiouronium salt method.
[d] B. p. 178–180°, 1–2 mm. pressure.

Preparation of the Lead Mercaptide.—To 0.1 mole of the appropriate mercaptan dissolved in 250 cc. of hot absolute ethanol was added 50 cc. of a 5 normal alcoholic lead acetate solution. An immediate precipitation of the lead mercaptide resulted. The mixture was digested on a water bath for a half hour and then filtered while hot. The precipitated material was washed with hot water and then with absolute ethanol and dried. The dried mercaptides were extracted with boiling ether and then dried in a desiccator (Table V).

TABLE V.—THE LEAD MERCAPTIDES

$(C_nH_{(2n+1)}S)_2Pb$

Compound	M. P., °	Yield, %	Pb, % Found	Pb, % Calcd
$(C_{10}H_{21}S)_2Pb$	98–100	88	37.3	37.4
$(C_{12}H_{25}S)_2Pb$	103–105	86	33.7	33.9
$(C_{14}H_{29}S)_2Pb$	104–105	86	31.0	31.1
$(C_{16}H_{33}S)_2Pb$	106–107	84	28.6	28.7
$(C_{18}H_{37}S)_2Pb$	109–111	82	26.4	26.6

The dried mercaptides were stored in an amber bottle and were fairly stable during the period of observation (two months). The mercaptans were regenerated as needed by treating the mercaptide with warm dilute hydrochloric acid and purified by distillation.

The water numbers of the mercaptans were determined in the manner described by Halpern and Zopf

(4) (Table VI). A correlation between the water numbers of the mercaptans and the hydroxy analogs with regard to carbon chain length was made (Fig. 1 and Fig. 2).

DISCUSSION

As a whole the mercaptan series caused rather little potentiation of the water absorption by petrolatum. The range in values obtained at the optimum concentration of the mercaptan was from 15 to 18. The hydroxy analogs ranged from 19 to 46 at their optimum concentrations. The water number at the optimum concentration continued to decrease slightly with the increase in the carbon chain length. The corresponding series of hydroxy derivatives, however, showed an increase in the water number with the increase of the carbon chain.

The optimum concentrations remained fairly constant in the range of 2 to 4%, and the decyl and tetradecyl mercaptans as well as the dodecyl and octadecyl mercaptans had the same optimum concentrations. The optimum concentrations of the corresponding hydroxy derivatives showed a decrease, with the increase in the carbon chain length, and ranged from 12.5 to 3%. The minimum, however, was at hexadecyl alcohol (4%) and there was a slight increase of the optimum with octadecyl alcohol (6%).

The poor emulsifying properties of the mercaptans may be explained, in part, by their rather limited tendency to associate. While it has not been conclusively established that hydrogen bonding does not occur with these *n*-alkyl mercaptans, it is generally accepted that the *n*-alkyl sulfhydryl compounds are nonassociated. The property of association or hydrogen bonding may well account, to some extent, for the superior emulsifying properties of the hydroxy analogs. The role of hydrogen bonding might be further established through infrared spectrum analysis. Further work is planned along these lines.

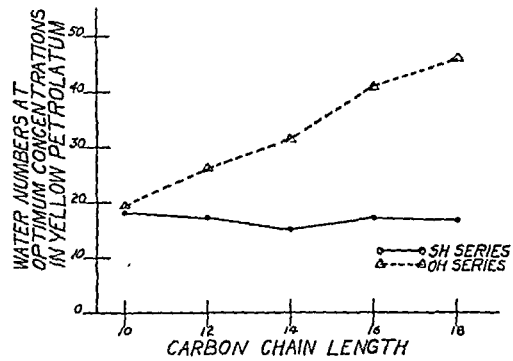

FIGURE 1

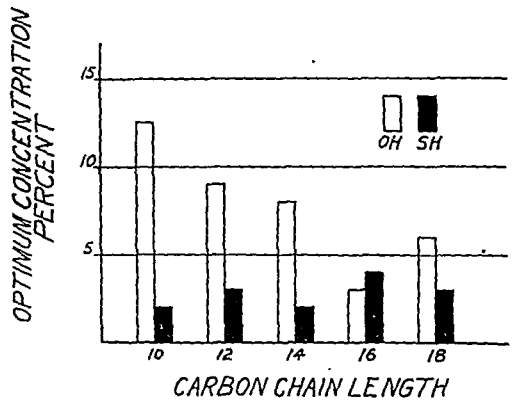

FIGURE 2

SUMMARY

1. The mercaptans with a carbon chain length from C_{10} to C_{18} were synthesized. The physical properties of these compounds and their intermediates were noted.

2. The water numbers of the mercaptans were determined and a correlation was made between the mercaptan series and their hydroxy-analogs.

3. The mercaptan series as a whole showed little ability to emulsify water in petrolatum. The range in water numbers for the mercaptan series was from 15 to 18 at the optimum concentration.

4. The optimum concentration of the mercaptans fluctuated slightly (from 2 to 4 per cent).

5. Since one contrasting difference between the series of mercaptans and the hydroxy analogs was the rather limited tendency of the sulfhydryl derivatives to form an associated complex, this might explain in part the rather superior properties of the fatty alcohols as emulsifiers.

TABLE VI.—THE WATER NUMBERS[a] OF THE *n*-ALKYL MERCAPTANS

Compound	Per Cent Concentration in Yellow Petrolatum[b]									
	1	2	3	4	5	6	7	8	9	10
$C_{10}H_{21}SH$	17	18	15.5	12.1	11.0	10.5	10.3	10.0	9.8	9.5
$C_{12}H_{25}SH$	14.2	16.1	17.3	14.3	13.1	11.1	9.8	9.6	9.4	9.1
$C_{14}H_{29}SH$	13.8	15.0	13.2	11.6	11.5	11.3	10.8	9.3	9.3	9.2
$C_{16}H_{33}SH$	13.2	15.1	16.3	17.1	14.2	13.1	12.0	10.3	9.6	9.2
$C_{18}H_{37}SH$	13.5	15.8	16.6	16.0	15.4	13.4	12.3	11.0	10.0	9.3

[a] Based on 100 Gm. of Mixture.
[b] Water number of the yellow petrolatum was 9.0

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Studies on the Hydrophilic Properties of Possible Ointment Base Constituents. IV. The Fatty Acid Esters of the Polyhydroxy Alcohols*

By ALFRED HALPERN† and NICHOLAS SQUEGLIA‡

The water numbers of a homologous series of fatty acid esters of polyhydroxy alcohols were investigated. The fatty acid esters of glycol, propylene glycol and glycerol (with the exception of glyceryl-mono-oleate) did not cause emulsion formation at the lower concentrations. Glycol-monostearate and propylene-glycol-monostearate afforded slight emulsification at concentrations greater than 10 per cent. The fatty acid esters of sorbitan and mannitan are excellent emulsifying agents. These agents produced the greatest increase in the water number of petrolatum of all the compounds studied. This study was limited to the W/O emulsions.

As PART of a study (1–3) of the hydrophilic properties of ointment base constituents in progress at these laboratories, a homologous series of fatty acid esters of the polyhydroxy alcohols was investigated. It was proposed (1) to develop a system of ointment base formulation that would enable the pharmacist to incorporate varying amounts of water or a solution of a medicament in accordance with the individual needs. A second phase of this study was to investigate the mechanisms of ointment formation and to determine the optimum concentration of the emulsifier that exerts the maximum effect as evidenced by the water number[1] of the fixed fat. The experimental agents used in this study have been investigated for emulsifying properties (5, 6). For the most part, these studies determined the effect of a fixed concentration of the emulsifying agent in the oil phase. No determination of the optimum concentration for emulsification activity was reported. Since the water number[1] of a mixture is an expression of the emulsification limits of the mixture, it was deemed important to determine these limits for a constant order of concentrations and note the optimum if it existed. A method of formulation would necessarily depend on these values and the flexibility of the use of these agents would be governed by these limits. It is emphasized that the preparations used are not to be construed as recommended ointment bases, nor is it proposed that the hydrated mixtures be interpreted as such. The mixtures are to be viewed as controlled experiments to determine the potentiation of the water numbers of the standard petrolatum due to the activity of the test compound.

EXPERIMENTAL

The water numbers of varying concentrations of ethylene glycol, propylene glycol, glycerin, sorbitol, a concentrated glucose syrup, and Simple Syrup, U. S. P. XIII, were determined in the manner described by Halpern and Zopf (1). All of these agents caused no potentiation of the water number of the petrolatum used. These agents all have a strong hydrophilic character and a significant absence of a hydrophobic group. It was expected that these agents would not evidence any emulsification properties because of the absence of the lipophile.

The fatty acid esters of the polyhydroxy alcohols used in this study were glycol-monostearate, pro-

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[1] The water number of a fixed fat was defined by Casparis and Meyer (4) to be the largest amount of water which 100 Gm. of an ointment base or fat will hold at normal temperature (20°).

pylene-glycol-monostearate, glyceryl-monolaurate, glyceryl-mono-oleate, glyceryl-monostearate, sorbitan-monolaurate,[2] sorbitan sesqui-oleate,[2] sorbitan-dioleate,[2] mannitan-oleate,[2] mannide-oleate,[2] and polyoxypropylene-mannitol-dioleate.[2]

The water numbers of these compounds were determined in the described manner (1) and the results were noted on Table I.

to litmus. The compounds prepared in this laboratory through the reaction between sodium stearate and epichlorhydrin and subsequent conversion to glycerol-1-stearate were neutral to litmus. The results reported in Table I were from neutral preparations.

The fatty acid sorbitan and mannitan esters greatly potentiated the water number of petrolatum.

TABLE I.—THE WATER NUMBERS OF THE FATTY ACID ESTERS OF THE POLYHYDROXY ALCOHOLS

Compound	Per Cent Concentration in Yellow Petrolatum[a]								
	1	2	3	4	5	7.5	10	12.5	15
Glycol monostearate[b]	No emulsion							14.1	14.2
Propylene glycol monostearate[b]	No emulsion						12.2	12.6	13
Glyceryl monolaurate[b]	No emulsion								
Glyceryl mono-oleate[b]	28	35	40	48	56	61	69	74	79
Glyceryl monostearate[b]	No W/O emulsion								
Sorbitan monolaurate	54	55	58	64	64	64	64	...	...
Sorbitan monopalmitate	64	69	76	84	92	128	150	180	241
Sorbitan monostearate	86	194	294	296	341	400	428	451	473
Sorbitan mono-oleate	360	440	605	740	900	1040	1180	1390	1470
Sorbitan sesqui-oleate	290	360	480	590	792	1010	1240	1340	1480
Sorbitan dioleate	491	482	601	716	800	981	1080	1110	1130
Mannitan oleate	295	361	400	481	580	651	1050	1200	1370
Mannide oleate	240	300	380	470	510	673	794	961	1121
Polyoxypropylene mannitol dioleate	Oil in water emulsion								

[a] Water number of petrolatum used was 10.1.
[b] Prepared in our laboratory. The *p*H of the 5 and 10% mixtures in water ranged from 6.8 to 7.2.

DISCUSSION

Glycol-monostearate and propylene-glycol-monostearate failed to produce W/O emulsions up to 10% concentration. The glycerol esters gave a rather poor potentiation of the water number when compared to the fatty acid esters of sorbitan and mannitan. This was rather surprising as the molecule had the usual requisites for an emulsifying agent, a hydrophilic group and a lipophilic group. Similar effects have been reported by Griffin (5).[3] No emulsion was obtained by him with glycol-mono-oleate and propylene-glycol-mono-oleate and glyceryl-monostearate (7). The trade literature suggests that small amounts of alkali be added to the aqueous phase for improved emulsification with some of these agents (8). This was done with a sample of 5% glycerol-monostearate in petrolatum. The addition of 0.1% alkali as sodium carbonate or sodium hydroxide to the aqueous phase resulted in a fairly good uptake of water by the mixture. The emulsion type was determined and was found to be O/W in contrast to the W/O type formed by the other esters *per se*. Since this study was confined to the mechanisms of the W/O emulsions, this effect was not further investigated in this study. It is of interest to note that some samples of commercially available glycol and glycerol esters from different sources and described as "self-emulsifiable" were found to be strongly alkaline in reaction

These compounds caused a water uptake up to 1200% (based on the 10% mixture). These agents appear to be superior to any of the agents tested. These compounds were unique in that the molecule contained both a hydroxyl group and an oxygen in an ether linkage. In addition to the groups contributing to the hydrophylic and lipophylic character of the molecule, there was also a cyclized nucleus resulting from anhydrization in their synthesis. The chemistry of these compounds is discussed in the trade publication describing their properties (9).

These agents apparently had no optimum concentration within the limits studied. The water number continued to increase with the increase in concentration. There appeared to be a leveling off of the rate of water number increase at about 10%.

It is particularly interesting to note the effect of the increase of hydrophilic groups on the molecule as evidenced by the water absorption of the mixture. It might be considered that the increase in the hydrophilic groups strengthens the bond between the hydrophile and the water at the same time weakening the attraction of the lipophile for the oil phase. The additional increase of the hydrophilic groups to the molecule so weakens the hydrophobe-oil bond that separation occurs. This factor may be demonstrated by the addition of polyoxyethylene groups to sorbitan mono-oleate. The resultant polyoxyethylene sorbitan mono-oleate gave no W/O emulsion in contrast to the excellent emulsifying properties of the sorbitan mono-oleate (7). Polyoxypropylene mannitol dioleate formed a fairly stable oil in water emulsion. The water number cannot be determined for an O/W emulsion.

The increased hydrophilic character of the fatty acid glycols in contrast to the fatty acid esters of the

[2] Furnished by the Atlas Powder Co., Wilmington 99, Del., through the courtesy of William C. Griffin of the Central Research Laboratory. These products are commercially available under the registered trade mark of "Arlacel," "Span," and "Tween."

[3] The emulsifying efficiency of glycerol-mono-oleate was reported (5) to be significantly greater than the value we found (Table I).

polyhydric alcohols might well explain the difference in emulsifying ability exhibited by these agents. In the W/O emulsions a determinant of emulsion efficiency might be the strength of the lipophile-oil bond. With the increased hydrophilic nature of the fatty acid glycols, the lipophile-oil relationship was weakened so that no W/O emulsion formed.

The saturated hydrated mixtures had rather poor pharmaceutical properties. There was a great loss of water, both by separation and evaporation, on storage at 50° for eight hours. The spreading of the hydrated mixtures was not satisfactory. While it is recognized that these mixtures were not intended to be used as ointment bases, their superior hydrophilic properties made them excellent starting materials for the formulation of bases that could be adjusted to the individual needs.

Preliminary experiments indicated that the separation of water from these mixtures could be minimized by reducing the hydration to 50–75% of saturation. The addition of small amounts of white wax improved the spreading qualities of the mixtures. The emulsifying agents were stable to dilute acid and dilute alkali at room temperature. They were compatible with such heavy metal salts as silver nitrate and mercuric chloride. The *p*H of the commercially available preparations ranged from 5.8 to 7.4. These agents apparently are nonirritating to the skin (10). When applied to the inner surface of the forearm for fourteen days no irritation was experienced. Work now in progress on the adjustment of these mixtures into suitable ointment bases will be reported at a later date.

SUMMARY

1. The water numbers of a homologous series of fatty acid esters of polyhydroxy alcohols was investigated.

2. The fatty acid esters of glycol, propylene glycol, and glycerol caused no emulsion formation at the lower concentrations, with the exception of glyceryl-mono-oleate. Glycol-monostearate and propylene-glycol-monostearate afforded slight emulsification at concentrations greater than 10 per cent.

3. The fatty acid esters of sorbitan and mannitan are excellent emulsifying agents. These agents produced the greatest increase in the water number of petrolatum of all the compounds studied.

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Color Reactions for Copalquin Bark Extract*

By JACOB A. SCHULDINER†,‡

The source, preparation, and use of copalquin bark extract as an adulterant and substitute for raw and smoking opiums is discussed. A series of qualitative tests are used to distinguish it from crude or smoking opiums and for the detection of admixtures.

MANY adulterants have been found in raw and smoking opiums. Recently a new one was alleged to have come from Mexico. This product, so-called "gum copalquin," has the odor, color, consistency and general appearance of opium. Its principal use has been for the adulteration of smoking opium. It has also been sold both as raw and as smoking opium without the admixture of genuine opium.

It has become necessary to distinguish this material and its mixtures with opium products from the pure narcotics. In order to accomplish this, the source material was identified, the method involved in its production was discovered, and a series of chemical tests for its identification and differentiation from opium were worked out.

SOURCE OF MATERIAL AND PREPARATION OF THE EXTRACT

While on a trip into Mexico, the author obtained authentic source materials from which gum copalquin is made. He was also able to confirm the identification of the substance as bark from the copalquin tree, *Coutarea pterosperma* (1, 2).

An authentic sample of the bark of *Coutarea pterosperma* was coarsely ground. The powder was placed in a large fritted glass funnel and extracted with cold distilled water. To aid the flow of extract a little vacuum was applied occasionally.

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The extraction was continued almost to completion, as indicated by the light color of the final extract.

After considerable experimentation it was discovered that when the odorless extract was treated with small portions of powdered hydrated lime and boiled to the proper consistency, a product was obtained which had the characteristic odor and appearance of smoking opium. The alkali and heat caused the formation of free volatile substances which have characteristic amine-like odors. These substances are probably formed either by hydrolysis and decomposition of proteins or by their release from amine-like salts present in the bark extract.

From the identification of the source of this material and its method of manufacture, it has been concluded that it would be more accurately described as copalquin bark extract.

SPECTROGRAPHIC ANALYSIS

Spectrographic analysis of copalquin extract ash indicates that the pure extract could probably be distinguished from Mexican raw or smoking opiums. However, the differences are not so specific that this purpose could be accomplished with mixtures of copalquin bark extract with smoking opium, raw opium, or opium residues.

PROCEDURE IN TESTING FOR ADULTERATION

A series of color tests were made with various reagents. These tests were on a group of fifteen authentic raw opiums from a wide variety of known sources; on several samples of gum copalquin; and on seizures of alleged Mexican smoking opiums, two of which were suspected to contain copalquin bark extract.

The reagents used in the tests were:

(1) Concentrated NH_4OH
(2) Sodium hydroxide 1.0% in H_2O
(3) $Ca(OH)_2$, a saturated H_2O solution
(4) Concentrated H_2SO_4
(5) Concentrated HCl
(6) Phosphoric acid 85%
(7) Ferric ammonium sulfate, dilute solution in H_2O
(8) Lafon's reagent: 1.0 Gm. of ammonium selenite in 20 ml. of concentrated H_2SO_4.
(9) Iodine solution in petroleum ether. A few crystals of iodine dissolved in 50 ml. of extraction-grade petroleum ether..

These tests with the noted exceptions (ferric ammonium sulfate and iodine in petroleum ether) were made by adding the reagent directly to a small portion of the solid material in a test tube.

Gum copalquin is readily soluble in cold alkalies with the exception of 50% sodium hydroxide. The solution in alkalies is a clear, deep brownish yellow color. Opiums are usually much less soluble and the resulting color is lighter and often a muddy suspension is present.

Concentrated sulfuric acid gives a pink to deep red color with gum copalquin. When this is carefully diluted with water, the dilution is pink. Opiums give yellow to brown colors with concentrated sulfuric acid

Concentrated hydrochloric acid gives pink to deep magenta shades with copalquin extract. With opiums, deep yellow colors are produced.

Phosphoric acid 85% gives characteristic pink to magenta colors with copalquin bark extract. With opiums, the colors are straw to deep yellow.

Ferric ammonium sulfate gives distinctive greenish brown precipitates with copalquin extract dissolved in distilled water. With opiums, the color is red-brown.

Lafon's reagent gives a red-brown color with copalquin extract and greenish brown with opiums.

Raw opiums give an orange color with iodine dissolved in petroleum ether.

The samples are dissolved in petroleum ether and filtered. The iodine reagent is added to the filtrate. Old, oxidized samples sometimes fail to give this reaction. If the hard outer portion is discarded, the unoxidized inner portion will usually respond to the test. With copalquin extract and smoking opiums, both of which are water extracts, the original purple color of the iodine reagent remains unchanged.

Mixtures of copalquin bark extract and opium are detected by the following criteria:

(1) Morphine content.
(2) Pink and red colors with H_2SO_4, HCl, and H_3PO_4, indicate the presence of copalquin bark extract.
(3) Increased solubility in alkaline reagents and deep yellow color often is a sign of copalquin extract adulteration.
(4) Mixed color reactions when compared with the color reactions of authentic copalquin extract and opium products are indicative of adulteration.
(5) The absence of morphine coupled with an orange color with the iodine in petroleum ether would indicate a mixture containing opium residues.
(6) Raw opium, in mixtures with copalquin extract, is indicated by the orange color with petroleum ether-iodine reagent, if morphine is also found present.
(7) Smoking opium, in mixtures with copalquin extract, is indicated by the morphine content coupled with a negative (purple) color with petroleum ether iodine solution.

CONCLUSIONS

Gum copalquin has been identified by means of authentic materials and chemically as an alkalized water extract of the bark of *Coutarea pterosperma*. The method of preparation of the extract has been determined. A series of tests have been evolved, which, combined with a quantitative determination of the morphine content, can be used to prove the adulteration of various opium products with copalquin bark extract.

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Note

An Inexpensive Pen for Kymographic Recording*

By THOMAS J. HALEY

THERE are times when those who work with isolated smooth muscle preparations desire, for recording muscular contractions, some method which does not employ the smoked kymograph drum. Several expensive ink-writing instruments are available but their continuous use requires more than the usual amount of care to insure a continuous flow of ink to the recording surface. A simple glass tip ink writer has also been used, but this is subject to breakage.

For the last two years this author and several of his colleagues have employed the commercially available "Beta" fountain pen[1] for recording both smooth muscle contractions and respiration in anesthetized animals. The pen is attached to the smooth muscle lever by a small drop of solder or plastic cement. It may also be attached by filing small holes in its supporting arm and inserting the muscle lever therein. If necessary a reduction in the weight of the simple type lever arm may be accomplished by using a hollow aluminum tube such as is used in making model airplanes. Under these circumstances the lever holder is reamed out to accommodate the larger diameter of the tube.

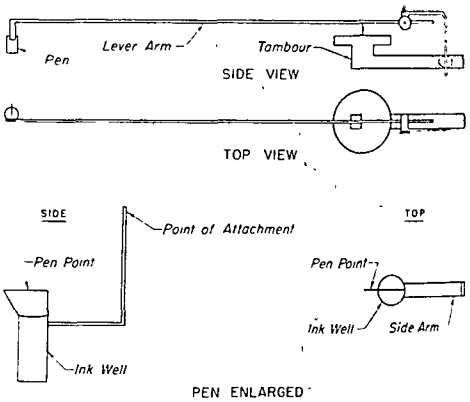

Fig. 1.—Pen and method for attachment.

This latter method is usually used when the pen is attached to the lever arm of the tambour used to record respiration. See Fig. 1 for a diagram of pen and method of attachment.

It is unfortunate that the weight of this simple pen prevents its use in recording blood pressure with a mercury manometer. Any attempt to use it for that purpose usually results in the damping of the pressure wave and no record. However, the pen might be useful with a Harvard membrane manometer.

* Received October 18, 1948, from Medical School, University of California at Los Angeles.

[1] American Recording Chart Co., 3113 East 11th Street, Los Angeles 23, Calif.

Book Reviews

The Basis of Chemotherapy. By THOMAS S. WORK and ELIZABETH WORK. Interscience Publishers, New York, 1948. xx + 435 pp. 14 x 22 cm. 42 illus. 38 tables. Price $6.50.

Beginning with his interest in the distribution of dyestuffs in the animal body in 1885, and culminating with the discovery of "606" in 1911, the works of Ehrlich form the true foundation for chemotherapy. After the discovery of arsphenamine there followed an intense search for chemotherapeutic agents capable of attacking bacteria as well as protozoa. Indifferent success in this area almost caused the field to be abandoned until the spectacular sulfonamides arrived on the scene. This rebirth brought with it an intensified study of the mechanism of drug action resulting in the competitive inhibition concept, so applicable to fields like the antihistaminics as well as infectious diseases.

Because of the intensified interest in chemotherapy during the last fifteen years or so, it is fitting that someone should review the field at this time. This has been accomplished by the Doctors Work who have succeeded in covering the field with a fair measure of thoroughness without the narrow-

ness of simply recording factual data. Consistently throughout the book is found a stimulating viewpoint.

After a very excellent introductory chapter on the history of chemotherapy, the author discusses cell metabolism, the biochemistry of essential metabolites and enzyme inhibition. These chapters form an excellent prologue to those following on drug antagonism, drug resistance, and the relation of structure to the activity of drugs.

This well-developed treatise will widen the perspectives of all workers interested in the hybrid field of chemotherapy.

Pharmacology. 3rd edition. By J. H. GADDUM. New York, 1948. Oxford University Press. xvi + 504 pp. 13 x 22 cm. Price $8.

The third edition of this text follows the same pattern as previous editions. Characteristic of the main additions and changes are folic acid, action of drugs on endocrine glands, drugs acting against acetylcholine, epinephrine, and histamine, anticoagulants, BAL, insecticides, detergents, sulfonamides, streptomycin, and penicillin.

Due to brevity, this text has limitations in the teaching of practical pharmacology to physicians and pharmacists. To those interested in experimental pharmacology, it is a very useful book, however. Of particular advantage is the modernized section on the use of statistics in bioassay.

The Biological Standardization of the Vitamins. 2nd edition. By KATHERINE H. COWARD. The Williams and Wilkins Co., Baltimore, 1947. vii + 224 pp. 13.5 x 23.5 cm. Price $5.

The Biological Standardization of the Vitamins is divided into two parts. Part I is a practical portion of the book. After two chapters dealing with general principles, the bioassays of the individual vitamins are discussed. This portion of the book terminates with a chapter on the interdependence of the vitamins.

Part II is more theoretical in nature and is concerned with a statistical treatment. In separate chapters the standard deviation, factors influencing the accuracy of vitamin determinations, the limits of error, and improvement of bioassays are discussed here. Considerable revision, based on research by Irwin, Bliss, Fieller and others, is in evidence.

Specialists in the field will find much of interest in this book.

Textbook of Pharmacognosy. 6th edition. By HEBER W. YOUNGKEN. The Blakiston Company. Philadelphia, 1948. 1063 pp. 15 x 23 cm. Price $8.50.

Perhaps no other division of the medical sciences has advanced more in the last decade than that dealing with the drugs of vegetable and animal origin. The introduction of antibiotics and vitamins, the "rediscovery" of a few drugs, such as pareira, cocculus, and podophyllum, and the purification of more of the plant constituents, such as glycosides and alkaloids, are reasons for revisions of works on pharmacognosy. A revision is also convenient, if not necessary, to keep pace with the Pharmacopœia.

The cutting off of supplies of cinchona from Java by World War II stimulated search for and the finding of many sources in Central and South America. It also encouraged experimental growing of cinchona in the Western Hemisphere.

The discovery and history of cinchona in the seventh century, while dealt with briefly by this author, adds much interest to the study of such an important drug. Cinchona and quinine production undoubtedly involve the application of more sciences than any other crude drug. Dr. Youngken has brought these topics up to date in a very usable form.

Allergy and pollen have been given the attention they deserve in a textbook of pharmacognosy.

It is thought that the discussions on vitamins should be much more complete than those found in this text.

Digitalis lanata is past the experimental stage and it deserves the attention given it in this book. This unofficial species of Digitalis, with actions quite similar to those of the official species, and with two to five times its potency, enables the physician to make a wider selection of heart tonics. The importance of Digitalis lanata is shown by the fact that two of its glycosides were introduced into U. S. P. XIII.

The increased importance of endocrine glands and their hormones justifies the enlargement of this section of the book, adding valuable illustrations and lengthy descriptions of both the whole and powdered glands. While the student will study the pharmacology of the endocrines later in his curriculum, their inclusion in the course in pharmacognosy enables the student to study them as a distinct group of drugs and compare them with other animal drugs as to origin, constituents, and other details.

Many authors of textbooks fail to realize the value of the history of their subject as a stimulant to the student's interest. Professor Youngken has a very good but much condensed history of pharmacognosy which might well be enlarged to include such topics as the very earliest writers on American drugs including Monardes, Martin de la Cruz, and David Schoepf.

It is suggested that Chapter III be divided into one chapter on antibiotics and one on bacterial biological products, and that each have an introduction to bring out the point that the inclusion in medicine of these groups was a great stride in the advance of medicine. The introduction to the second of these chapters might be greatly enhanced by a discussion of Louis Pasteur, who was the Father of Immunology.

The author has treated the antibiotics in a very thorough way. It would be unwise to attempt to list all of the antibiotics here or to give a complete discourse on any one, but the author has included concise monographs on penicillin, streptomycin, tyrothricin, chlorophyll, and bacitracin.—CHALMERS ZUFALL.

Lectures on the Inorganic Nutrition of Plants. Second Printing. By D. R. HOAGLAND. The Chronica Botanica Co., New York City. Stechert-Hafner, Inc., 1948. 226 pp. 14.5 x 23 cm. Price $4.50.

The subject of this volume is Dennis R. Hoagland's plant nutrition lectures which were presented

at the recent Prather Lectureship series at Harvard University. The material is abundant in data of interest to students and teachers alike in the important functions and mechanisms by which inorganic elements affect plant life. Seven lectures are edited in splendid style covering first a survey of plant nutrition problems, the role of the nutrients, potassium and calcium, and the micronutrients, zinc, manganese, cobalt, boron, copper, iron, and molybdenum, in plant functions. This is followed by lectures on special phases of plant physiology and plant chemistry. Included in the latter series are the absorption and accumulation of salts by cells, movement of inorganic solutes in plants, and various plant nutrition factors involved in water and sand culture media. Of great significance to the pharmacist is lecture Number Six. Herein is a discussion of some biochemical problems associated with salt absorption, organic acids, nitrogen, protein and ion accumulation since these factors influence chemical processes throught to be important in plant biosynthesis. The author might have speculated further to the advantage of the plant chemist. The review of the Krebs cycle and the allusion to the importance of ammonia in plant amide synthesis are helpful.

Only very general observations are offered relative to the field of radio active isotopes as tools in plant nutrition problems. In fact the reader finds himself wishing for more on this subject, especially data dealing with the methods. Nevertheless many useful references are included. In fact each lecture is well referenced at its conclusion.

The series is accompanied by twenty-eight plate-photographs showing experimental plant growth observations covering certain aspects of the text. Most of these appear to be of material which is under experimentation in the Division of Plant Nutrition Laboratories, University of California. The pharmacognosist or plant chemist will gain much from each of Hoagland's lectures and, more important still, he will find himself continuously in a speculative mood during his reading.—HEBER W. YOUNGKEN, JR.

International Rules of Botanical Nomenclature. By W. H. CAMP, H. W. RICKETT, and C. A. WEATHERBY. Waltham, Massachusetts: The Chronica Botanica Company. New York City: Stechert-Hafner, Incorporated, 1948. 120 pp. 17 x 26 cm. Price $3.50.

Plant taxonomists and allied botanists have long had need of an up-to-date compendium of the International Rules of Botanical Nomenclature. In this text the authors have assembled those major articles of the "Rules" initiated during the Botanical Congress at Amsterdam (1935) together with those recommendations for further changes in nomenclature which have since been proposed by recent International Botanical Congresses. The text is not intended to be an official printing of the format of the third (1935) edition of the "Rules." Nevertheless it follows the official form closely. Even the casual taxonomist will find stimulation in the many proposed recommendations. The index of conserved and rejected generic names in Appendix III is a decided feature.—H. W. YOUNGKEN, JR

A Catalogue of Insecticides and Fungicides. Volume II. Chemical Fungicides and Plant Insecticides. By DONALD E. H. FREAR. The Chronica Botanica Co., Waltham, Mass., 1948. xii + 153 pp. 19.5 x 27 cm. Price $5.50.

For review of Volume I see THIS JOURNAL, 37, 214 (1948).

Dr. Frear set up a project at Pennsylvania State College to catalogue all substances which have insecticidal and fungicidal properties as recorded in the literature. Like Volume I, Volume II gives the chemical names, synonyms, and complete formulas as well as the results of tests on insects and fungi for chemical fungicides and plant insecticides. References and author indexes, patent lists, and a complete index of compounds listed in both volumes are present. Keys to the literature of this type are useful to both chemists and entomologists.

Elseviers' Encyclopedia of Organic Chemistry. Edited by F. RADT. Series III, Carboisocyclic Compounds, Volume 12B, Part I, Naphthalene. Elsevier Publishing Company, New York, 1948. xxx + 344 pp. 16 x 25.5 cm. Price: Subscription price $24; series price $28; single price $32.

For some time many chemists have wished for an English "Beilstein" which would augment, and, to a certain extent replace, the original. This is one of the early volumes of an encyclopedia to be produced to answer such a need. The compilers started with a volume other than "I" in order to make available literature not so readily accessible in "Beilstein" at the present. This volume, for example, covers the general literature up through 1944 and the literature concerning the structure of compounds through 1948.

The editors have kept in mind the fact that the organic chemist no longer works in isolation, and have introduced data of interest to the physicist and those working in the many fields of biology.

The arrangement of compounds is simple and the system is set up so that compounds most closely related are placed together. In connection with each compound, its additive derivatives are described—the only exception being where the derivatives belong to a very important heterocyclic ring system.

The generous use of tables makes it easy for the reader to get information quickly. For example, on Pages [illegible] is a very complete tabulation of the solubility of naphthalene in a variety of solvents at different temperatures. The table is usefully arranged and well documented.

Wherever possible information is given as to physical properties of the compound, its chemical preparation, its stability, its physical properties, physiological action, and the preparation of principal derivatives.

Each volume [illegible] complete subject and formula index.

The typograph[illegible] excellent and the type [illegible] conducive to easy reading. Like "Beilstein," [illegible] value of this work lies in its completeness. [illegible] other volumes must maintain this high standard [illegible] become available [illegible] research libraries and laboratories will have a [illegible] of chemical information easily accessible.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Acetic Acid as an Irritant in Pyrethrum Extracts*

By ROBERT L. FRANK† and ROBERT L. McGEACHIN†

Highly active dermatitis-producing concentrates have been obtained from pyrethrum flowers by ether extraction and by steam distillation, followed by further fractionation. The most active fractions have been found to be rich in acetic acid, previously unreported in pyrethrum. The suggestion is made that acetic acid contributes to the dermatitis-producing properties of pyrethrum extracts.

IT IS well known that certain individuals working with pyrethrum extracts or flowers have been subject to a dermatitis presumably caused by substances contained in the flowers (1). Martin and Hester (2) obtained active concentrates by ether extraction and by steam distillation of the ground flowers. Feinberg (3) reported that 46 per cent of a group of 225 patients sensitive to ragweed pollen gave positive cutaneous reactions with pyrethrum extracts. Sweitzer (4) has reported a much smaller incidence of sensitive individuals.

The fact that the irritant material is volatile with steam indicates a fairly low molecular weight for the active principle and has encouraged us to investigate further the chemical nature of the dermatitis-producing constituents of pyrethrum. Fractions having irritant properties in sensitive individuals have been obtained in our work from pyrethrum flowers by two methods: ether extraction and steam distillation, both followed by fractional distillation. Various fractions from these experiments were submitted to Dr. A. Rostenberg of the University of Illinois Medical College, Chicago, Ill., and to Dr. S. Mackoff of the University of Minnesota General Hospital, Minneapolis, whose skin tests on sensitive individuals are summarized in Table I.

Of the fractions obtained by ether extraction and distillation, one (Sample No. 7 of Table I) seemed of particular interest because it was not only one of the most potent, but must have consisted entirely of low-boiling material, since it was not condensed in the ice-cooled receiver during distillation of the oleoresin at 2 to 3 mm. pressure. It was collected instead in the dry-ice trap. Investigation showed this material to

* Received August 30, 1948, from the Noyes Chemical Laboratory, University of Illinois, Urbana, Ill.

† We are indebted to the United States Public Health Service for funds which made this work possible. We also wish to thank Dr. C. P. [illegible] Gormley-King Company [illegible] of pyrethrum flowers; [illegible] University of Illinois Coll[illegible] the University of Minnesota General Hospital, for finding and patch-testing pyrethrum-sensitive individuals; and Mr. Joseph Dailey for helpful suggestions during the course of the work.

TABLE I.—CUTANEOUS REACTIVITY OF FRACTIONS FROM PYRETHRUM FLOWERS

Sample	Description of Sample	Reaction: Patient 1	Reaction: Patient 2
Tests by Dr. A. Rostenberg, Jr.[a]			
1	Ground pyrethrum flowers	++++	±
2	Ether-soluble oleoresin from pyrethrum flowers	++++	+++
3	Crystals (m. p. 198°) from ether extract before distillation (probably chrysanthin)	±	–
4	Ground pyrethrum flowers after ether extraction	?	–
5	Alcoholic extract of residue after distillation of oleoresin obtained by ether extraction	–	–
6	Distillate from ether extract (material volatile at 2–3 mm. pressure with bath temperatures up to 250°); n_D^{20} 1.4208	+++	+++
7	Low-boiling distillate from distillation of oleoresin obtained by ether extraction (collected in dry-ice trap); n_D^{20} 1.3890	++++ (large blister)	++++
8	Oil from ether extract of steam distillate of pyrethrum flowers	++++	++
9	Same as no. 8	++++	±
10	Oil from ether extract of steam distillate of pyrethrum flowers, in which the steam distillate was made alkaline with sodium bicarbonate before extraction	++++ (large blister)	++++
Tests by Dr. S. Mackoff[b]			
11	Oil from ether extract of steam distillate of pyrethrum flowers, the distillation carried out with return of water to still	–	....
12	Distillate from ether extract of steam volatile oil, b. p. 80–84°/5 mm.; n_D^{20} 1.4855	–	....
13	1% aqueous acetic acid	–	....

[a] Tests carried out by Dr. A. Rostenberg, Jr., of the University of Illinois College of Medicine.
[b] Tests carried out by Dr. S. Mackoff of the University of Minnesota General Hospital on six patients who gave positive reactions with pyrethrum flowers.

contain approximately 30 per cent acetic acid, characterized by boiling point, refractive index, Duclaux constants, and the formation of a crystalline *p*-bromophenacyl ester.

The occurrence of acetic acid in pyrethrum flowers is of interest because it has apparently not been previously reported.[1] It is of further interest because a 30 per cent solution of acetic acid is irritating to the skin of most persons.[2] Thus arises the natural suggestion that some of the irritation of individuals to pyrethrum oleoresins is due to acetic acid, concentrated during the extraction process.

Examination of Table I brings out the fact that the active fractions are just those in which acetic acid would be concentrated during their preparation. Even Sample 10, an ether extract from the steam-volatile oil made alkaline before extraction, must contain acetic acid, since it can be readily demonstrated that acetic is a weak enough acid to be extracted by ether from an aqueous alkaline medium.

The same statement, i.e., none of the irritant fractions described so far can be said with certainty to be devoid of acetic acid, appears to hold true for the fractions tested by Martin and Hester (2).

It is not to be inferred from these findings that acetic acid is the main allergenic principle in pyrethrum flowers. Such a conclusion is denied by the fact that ground pyrethrum flowers alone, unconcentrated in acetic acid, give a strong reaction in some individuals.[3] Further, the extreme effects on J. T. Martin of the extracts studied by Martin and Hester can hardly be ascribed to the main ingredient in household vinegar. On the other hand, the nonspecific skin irritation caused by acetic acid is similar in appearance to that evoked by the specific allergen of pyrethrum flowers, and we believe that a number of the extracts tested by Martin and Hester and by others probably contained enough acetic acid to act as a primary irritant. This may account for the disturbing observation of Martin and Hester in their excellent report that all their fractions, obtained by various means, gave positive reactions, though of varying intensities, and that all these fractions had, to different degrees, the sour odor of pyrethrum flowers.

The relative amounts of acetic acid in various extracts may also explain the divergence in the reported incidence of pyrethrum dermatitis

[1] The odor of dried pyrethrum flowers is probably partly due to the presence of acetic acid. The odor of very dilute solutions of the latter markedly resembles the faintly sour smell of pyrethrum.

[2] Experiments in this Laboratory have indicated that most normal individuals produce a red welt after an exposure of one hour to a gauze patch moistened with 10% aqueous acetic acid.

[3] A 1% aqueous acetic acid solution gave a negative reaction on a patient sensitive to the dried flowers (Table I, Sample 13).

(3, 4), mentioned in the introduction to this report. Studies with the flowers themselves, however, are not likely to be influenced by acetic acid, since before extraction the latter is present in amounts of less than one per cent.

Thus far the complete removal of acetic acid from our active concentrates has resulted in total loss of cutaneous activity.

EXPERIMENTAL

Ether Extraction of Pyrethrum Flowers.—Twenty pounds of powdered Kenya pyrethrum flowers (Table I, Sample 1) were extracted (in one-pound lots in a Soxhlet extractor) with ethyl ether (2 L. of ether per pound of flowers) to give an ether solution having a dark green color. The ether was removed slowly by distillation at atmospheric pressure. When approximately four-fifths of the ether had been removed, a small quantity of colorless rhomboid crystals separated. The ether solution was decanted from the crystals, which were then removed, washed well with cold ether, and air dried (Table I, Sample 3). From their melting point, 198° (201° corr.), they were judged to be chrysanthin (5).

The remainder of the ether was then removed by distillation at atmospheric pressure and further by evacuation to 20 mm. pressure. The residue was a dark green semisolid oleoresin weighing 1400 Gm. (Table I, Sample 2).

This residue was then subjected to distillation without fractionation through a short distilling head at 3–4 mm., with bath temperatures increasing to 250°. A fraction of 25 ml. was collected in a receiver cooled by an ice-bath (Table I, Sample 6); another 25-ml. fraction was obtained in a dry-ice trap connected to the receiver (Table I, Sample 7). The former fraction contained some water, which separated as a separate phase. The latter fraction was clear and colorless, but turned reddish brown on standing. It had a few crystals floating on its surface; these were removed, recrystallized three times from 50% aqueous ethanol and dried *in vacuo*, m. p. 48–50°.

Anal. Found: C, 83.10; H, 14.01. These were not further characterized.

The material in the dry-ice trap was found to be approximately 30% acetic acid, as described below.

Characterization of Acetic Acid.—A 1.0-ml. portion (sp. gr. 0.90; n_D^{20} 1.3745) of the liquid obtained above in the dry-ice trap was diluted with 15 ml. of water and titrated with 0.12 *N* sodium hydroxide, using phenolphthalein as indicator. It required 37.8 ml. to neutralize the acid, indicating, on the assumption that all the acid present was acetic, an acetic acid content of 29.9% in the fraction.

Three milliliters of the same material was diluted with 100 ml. of water. Ten milliliters required 9.9 ml. of 0.1 *N* potassium hydroxide for neutralization. On distillation successive 10-ml. portions required 6.8, 7.4, and 8.1 ml. of alkali. The Duclaux numbers for acetic acid are 6.8, 7.1, and 7.4.

The solutions of potassium acetate formed in the determination of the Duclaux numbers were combined, evaporated to dryness, and the potassium acetate fused. This salt was used to prepare a *p*-bromophenacyl ester, m. p. 84° (lit. (6), 85°).

Steam Distillation of Pyrethrum Flowers.—(1) One hundred and fifty grams of powdered pyrethrum flowers were steam distilled for approximately thirty hours (until no more oil could be detected in the condensate). The distillate was then saturated with sodium chloride and extracted with ethyl ether. The extracts were dried over anhydrous sodium sulfate and the ether evaporated to give 0.5 ml. of orange-yellow oil having a sour odor (Table I, Samples 8 and 9).

(2) Eight lots of 2000 Gm. each of powdered pyrethrum flowers were steam distilled, each lot being steam distilled about thirty hours (until no more oil could be detected in the liquid distilling). The apparatus used was such that the water layer from the distillate was continuously returned to the distilling flask. The oil was lighter than water so that it remained on the surface. The distillate from each lot was extracted with three 100-ml. portions of ether, the ether extracts from all the runs being combined and dried over anhydrous magnesium sulfate. The ether was then removed by distillation at atmospheric pressure and a bath temperature of 45–50°. When most of the ether had been removed, the temperature of the bath was raised to 80°, whereupon a volatile liquid distilled. This had an odor resembling that of acetaldehyde and gave a positive reaction with fuchsin-aldehyde reagent. The quantity was too small for further investigation. The liquid remaining was viscous; light orange in color; had a sour odor; and was distinctly acid to litmus (Table I, Sample 11). It is to be noted that this second means of steam distillation in which the water is returned to the flask fails to concentrate enough acetic acid to give a positive skin test. The yield was 16.7 Gm., or 0.1%, of the dry weight of the flowers.

This liquid was fractionally distilled through a small Podbielniak column to give 1.5 Gm. of crude acetic acid, n_D^{20}, 1.3841 (all the material distilling below 47° (10 mm.) and nine approximately equal fractions of b. p. 47–95° (5 mm.) having n_D^{20} ranging from 1.4856 to 1.4862. A middle fraction (Table I, Sample 12), although inactive as an irritant, was further characterized as follows: it was a viscous yellow oil, insoluble in water but soluble in ethanol and ethyl ether; sp. gr.$^{25}_{25}$, 0.948. It gave an acid reaction with litmus and had a neutral equivalent of 343. It decolorized bromine in carbon tetrachloride solution with no evolution of hydrogen bromide, and also decolorized alkaline potassium permanganate. It contained no nitrogen (Dumas), sulfur (sodium fusion), nor halogen (Beilstein test).

Anal. Found: C, 75.81, 75.74; H, 10.00, 9.91; C-Methyl: 12.0, 11.7%.

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Observations on the Mode of Action of Metrazol in Convulsive Doses*

By MAYNARD MURRAY, C. ROWELL HOFFMANN, and FRED R. SCROGGIN

An increase in the guanidine content of the blood of epileptics has been reported by Ellis (1). In a recent communication (2), it was shown that there was not only a slight increase in the basal level of guanidine-like substances in the blood of patients with epilepsy, but that there was a synchronous rise in the quantity of these substances in the blood with the convulsion (Fig. 1). We were interested to determine whether this rise in blood guanidine levels occurred in artificially induced fits as well as in spontaneous ones, i.e., whether the guanidine rise

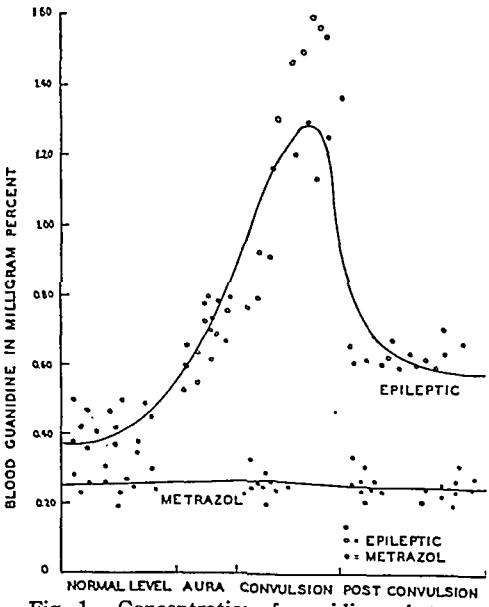

Fig. 1.—Concentration of guanidine substances in the blood in metrazol and epileptic convulsions.

was a result of the convulsion *per se*, or whether there was a difference in this biochemical reaction in the two types of fits.

The Effect of Metrazol Shock on Blood Guanidine[1] Levels

A group of ten patients at Longview State Hospital who were receiving metrazol shock therapy was studied. The same method for the determination of guanidine-like substances in the blood was employed as that used in the previous study on idiopathic epileptics. The normal blood guanidine level, and the levels at periods of one, fifteen, and sixty minutes after injection of metrazol in convulsive doses were ascertained in each case. The results of these determinations are given in Table I.

A comparison of the alteration in blood guanidine levels in spontaneous and in metrazol fits is represented graphically in Fig. 1. It will be seen that in this series of metrazol convulsions on ten patients all the values fill within the normal range, as reported by Andes and Myers (3), and that there was no rise in the blood guanidine levels in these fits comparable to that seen in spontaneous convulsions in idiopathic epileptics.

The Effect of Metrazol Shock on the Gonads

Investigators are generally agreed that many patients with involutional melancholia are improved markedly by metrazol shock therapy. This disorder is commonly considered to be due to a gonadal hormone insufficiency. Since it has been demonstrated by Marshall & Verney (4) that ovulation of

TABLE I.—GUANIDINE VALUE IN MG. % IN METRAZOL CONVULSIONS

Patient's No.	Normal Level	One Minute After Metrazol	Fifteen Minutes After Metrazol	Sixty Minutes After Metrazol
16	0.23	0.23	0.14	0.21
17	0.27	0.25	0.26	0.26
18	0.24	0.29	0.31	0.28
19	0.31	0.33	0.34	0.32
20	0.23	0.24	0.24	0.24
21	0.26	0.25	0.25	0.25
22	0.25	0.25	0.24	0.23
23	0.26	0.26	0.27	0.25
24	0.28	0.27	0.27	0.27
25	0.19	0.20	0.21	0.20

central origin through the anterior pituitary may be induced in the rabbit by electrical stimulation of either the cerebrum or the lumbosacral cord, and by convulsions produced by the intravenous injections of picrotoxin (5), the following question occurred: Does metrazol shock exert its beneficial therapeutic effect in these cases by acting directly upon the gonads, or upon gonadotropic secretions of the anterior pituitary gland?

An attempt to answer this question was made. Because of their peculiarly prompt response to estrogens and to gonadotropic anterior pituitary fractions, female rabbits of twelve to fourteen weeks of age were chosen as test animals, since any anterior pituitary gonadotropic principle would in all likelihood cause ovulation (6), while follicular activity would cause endometrial hyperplasia (7).

* Received Nov. 13, 1948, from the Physiology Laboratory, University of Cincinnati. This work was done in 1940 but interrupted by the authors' service with the Armed Forces.

[1] Throughout this article the term guanidine is used in lieu of guanidine-like substances.

Twelve female rabbits weighing between 2400 Gm., and 2800 Gm., were given intravenous injections of 0.20 Gm. of metrazol. Immediately the animals were thrown into violent convulsions, lasting between ten and fifteen minutes. With the exception of one animal, which broke several teeth as a result of spasm of the muscles of mastication, no injuries or fractures were observed. Following this, laparotomies were performed on the animals, in groups of three at twenty-four, forty-eight, seventy-two, and ninety-six hours, respectively. The ovaries, tubes, and uteri were removed. In no case did examination show changes in the ovaries such as congestion, enlargement, developing follicles or corpora hemorrhagica or leutes. A study of sections of the uterine mucosa showed no evidence of endometrial hypertrophy.

Another group of twelve female rabbits of the same age and weight range were given metrazol convulsions on successive days for seven days, and then forty-eight hours after the last convulsion, laparotomies were performed. In no case were ovarian changes noted, nor were any changes noted in the uterine mucosa when sections of this organ were studied.

CONCLUSION

Metrazol fits are not attended by a rise in the level of blood guanidine as are spontaneous convulsions in idiopathic epileptics, indicating a different chemical reaction in the two types.

Metrazol convulsions cause no changes in the ovaries or the endometrium of female rabbits indicating that this therapy, although effective in involutional melancholia, does not act in any demonstrable fashion on the anterior pituitary gland or the gonads.

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Qualitative and Quantitative Determination of Peroxides in Petrolatum*

By MILTON J. GOLDEN

A simple and accurate qualitative and quantitative Peroxide Test method is proposed for the determination of peroxides in petrolatums. The amount of peroxides in a petrolatum can be determined chemically by the oxidation of the ferrous ion to the ferric ion by the peroxides, utilizing the corresponding development of a pink or red color. Samples of petrolatum have been tested accordingly, and a table is given which compares the quantitative Peroxide Values of the petrolatums with their respective Peroxide Tests. A Peroxide Test of at least two hours is suggested as one of the minimum standards for petrolatums used in the manufacture of pharmaceuticals and cosmetics.

PETROLATUM, a substance closely related to White Mineral Oil, has become, in recent years, an important constituent of pharmaceutical and cosmetic preparations. In contrast to mineral oil, which is a liquid under normal conditions, petrolatum is semisolid at ordinary temperatures, consisting of a mixture of solid and liquid hydrocarbons. It is amorphous and microcrystalline and may be regarded as a colloidal system in which two phases are present, an external phase of solid wax and an oily internal phase.

In the selection of the proper type of petrolatum for a specific purpose, several important properties must be considered, namely fiber characteristic, melting point, and consistency. It seems that for most pharmaceutical and cosmetic preparations, a medium fiber is generally used. A short fiber petrolatum, characteristic of synthetic petrolatums and those refined by chemical treatment, is undesirable since it tends to produce a thin body and lacks the smooth consistency usually found with medium fiber. A long fiber petrolatum, on the other hand, produces a sticky and stringy finished product. In addition to the fiber characteristic, the melting point demands consideration and should be sufficiently high so that the finished preparation will not liquefy at slightly elevated temperatures. Furthermore, correct and uniform consistency is by far one of the most important factors in determining the suitability of a petrolatum.

The Pharmacopœia of the United States has recognized the importance of the purity of petrolatum and has, therefore, included tests for ash content, organic and mineral acids, alkali

* Received Nov. 8, 1948, from the Research Laboratories, McKesson & Robbins, Inc., Bridgeport, Conn.

and color, in addition to the properties already mentioned. It does not, however, include any specific test for determining stability.

As in the case with mineral oil (1), petrolatum is also known to develop peroxides upon heating and prolonged standing, which are objectionable and undesirable. It is important that petrolatums which are to be used in the preparation of pharmaceuticals and cosmetics should resist deterioration and be stable for a reasonable length of time in respect to odor, color, and development of peroxides. Moreover, this factor of stability is of major importance and particular interest to those pharmaceutical and cosmetic manufacturers who subject petrolatum to high temperature for various periods of time.

The so-called Peroxide Number method has been suggested for determining the amount of peroxides in petrolatums (2). However, various sources of error exist in this method. There may occur the reabsorption of the liberated iodine by the petrolatum and the oxidation of the reagents by air. Furthermore, the peroxides in the petrolatum do not seem to react readily with the potassium iodide. Consequently, the values obtained are not reproducible and reliable.

The purpose of this paper is to introduce a simple and accurate method for the qualitative and quantitative determination of peroxides in petrolatum.

EXPERIMENTAL

It has been shown in previous reports that the peroxide content of mineral oils can be determined chemically by the oxidation of the ferrous ion to the ferric ion by the peroxides formed in heat-treated oils (1, 3). This oxidation can be measured colorimetrically.

In the Peroxide Test for Mineral Oils (1), a colorless test solution of ferrous sulfate, ammonium thiocyanate, sulfuric acid, reduced iron and iron wire is added to samples of mineral oil heated in a 300° F. oil bath for various time intervals and the developing color observed. The color varies from colorless to dark red, depending upon the amount of peroxides formed during the breakdown of the oil. The first sample exhibiting a pink coloration after shaking with the test solution is regarded as the Peroxide Test value expressed in minutes.

These facts and reactions have been used in the development of the following qualitative and quantitative procedure for detecting and estimating the amount of peroxides formed in petrolatums.

The consistency and structure of petrolatum necessitated the use of a solvent which had to be miscible with the petrolatum and not with the test solution. Several organic solvents have been tried and found to be unsuitable due to their miscibility with the acetone contained in the test solution. Only mineral oil possesses the required properties. The mineral oil was of high purity and had a Peroxide Test of at least thirty-five minutes.

In the Peroxide Test method, samples of petrolatum are heated at 250° F., the temperature equivalent to 15 pounds of steam pressure, for various time intervals from one-half hour to several hours and then mixed with mineral oil, heated to the same temperature in the ratio of 2:3. The petrolatum mineral oil mixture is then cooled to 170° F. Subsequently, the test solution, consisting of ferrous sulfate, ammonium thiocyanate, sulfuric acid, iron and iron wire, is added, and the change in color of the lower test solution layer from pink to darker pink or red, depending upon the amount of peroxide formed, is observed and measured. Since there are always some peroxides present in petrolatum, the formation of a pink color in the test solution layer with unheated samples is to be expected.

Apparatus.—One-liter round-bottom flask and reflux condenser; test tubes $^3/_4$ x 6 inches; constant temperature oil bath regulated at 250° F. ± 1° F. glass-stoppered reaction flasks with greaseless stopcock, according to Connor and Staub's vitamin B fluorometric method; centrifuge; Klett-Summerson photoelectric colorimeter with filter No. 56, having a mean transmission of 560 mμ, and special test-tube cells.

Materials.—Ferrous Sulfate C. P.; Ammonium Thiocyanate, C. P.; Iron by Hydrogen, Merck (reduced iron); Iron Wire No. 30, for standardization Acetone U. S. P.; 3% Hydrogen Peroxide Solution

Preparation of Reagent.—In a round-bottom wide-mouthed flask, 2 Gm. of ferrous sulfate is dissolved in 100 cc. of distilled water containing 2 cc concentrated sulfuric acid. Two hundred cubic centimeters of acetone and 100 cc. of a 2% aqueous solution of ammonium thiocyanate are then added to the above solution. Iron powder 0.2 Gm. and 6 inches of No. 30 iron wire are finally added to reduce any trace of ferric ion present. The resulting mixture is refluxed on a steam bath until the red color of the solution has disappeared. This reduction process takes about two hours, it being necessary to continue refluxing to insure lack of color.

Fresh solution is prepared on the day of the test It is possible, however, to preserve it in a colorless state by careful storage in a carbon dioxide atmosphere.

Since it is necessary to avoid any contact with air, a dispensing method has been devised. A siphon is passed into the upper end of the reflux condenser through a two-hole cork stopper. The second hole is fitted with a glass stopcock, which serves as a vent. Carbon dioxide is admitted through the vent to inaugurate the flow of the solution, the pressure forcing the liquid into the siphon at the end of which is attached a glass stopcock for the withdrawal of the solution.

Qualitative Procedure.—Petrolatums to be tested are immersed in a constant temperature oil bath at 250° F. ± 1° F. Two-cubic centimeter samples of this heated petrolatum are removed after one-half hour and at hourly intervals thereafter and then mixed with 3 cc. of mineral oil heated to the same temperature in individual test tubes. The petrolatum-mineral oil mixture is then cooled to 170° F. The air in the test tubes is replaced with carbon dioxide and 5 cc. of the test solution is added. The air in the tube is again replaced with carbon dioxide. The tube is tightly stoppered with a new cork stopper and shaken by hand for fifteen seconds. The

formation of additional peroxides in the petrolatum is detected by the development of a darker pink to red coloration of the bottom layer in the test tubes. The first tube exhibiting a change in color from pink to darker pink or red after shaking with the test solution is regarded as the Peroxide Test Value expressed in hours.

Quantitative Procedure.—The colorimetric Peroxide Test Method (3), recently proposed for determining the amount of peroxides formed in mineral oil, has been modified and adopted for petrolatums. Samples of petrolatums to be tested are immersed in a constant temperature oil bath at 250° F. ± 1° F., the temperature equivalent to 15 pounds of steam pressure, and then mixed with mineral oil heated to the same temperature in the ratio of 2:3. A 10-cc. sample of the heated petrolatum-mineral oil mixture, cooled to 170° F., is pipetted into a glass-stoppered reaction flask which has been marked to indicate 10-cc. and 20-cc. filling. The air in the flask is replaced with carbon dioxide, and 10 cc. of the colorless test solution is added. The flask is then tightly stoppered with the glass stopper, shaken by hand for one minute, and centrifuged for three minutes. The lower test solution layer which will then vary in color from pink to dark red is transferred by means of the greaseless stopcock from the reaction flask to a cork-stoppered special Klett test-tube cell, in which the air has also been replaced with carbon dioxide. As a control, 10 cc. of the mineral oil, heated to 250° F. and then cooled to 170° F., is treated similarly and run parallel with the heated sample. The test-tube cells, containing the sample and control, are then placed immediately in the Klett colorimeter, and the amount of pink or red color formed is measured using the Klett filter No. 56. The difference in scale readings between the sample and control gives the reading for the oxidation in the test solution by the peroxides in the melted petrolatum. With certain types of petrolatums, the turbidity of both control and sample must be considered in order to obtain reproducible values. The turbidity of both solutions is measured with the Klett filter No. 66, and the values obtained are then subtracted from the original readings.

Prior to the above-described procedure, the Klett photoelectric colorimeter is adjusted until a zero reading is obtained with the colorless test solution and then standardized with various concentrations of hydrogen peroxide by measuring the amount of color corresponding to various quantities of peroxide. A freshly prepared 3% hydrogen peroxide solution is diluted 1–100, and its peroxide content is determined according to the U. S. P. assay method with 0.01 *N* potassium permanganate solution. Very small volumes of this diluted peroxide solution, having a concentration range of 1×10^{-6} to 1×10^{-4}, are added to 5-cc. portions of the colorless test solution in cork-stoppered Klett test-tube cells, and the amount of pink or red color formed is measured in the colorimeter.

Hydrogen peroxide concentrations are plotted against scale reading, and a standardization curve is obtained with increasing concentrations of peroxides. The resulting graph facilitates the determination of the amount of peroxides in any sample of petrolatum by evaluating the concentration corresponding to the difference in scale readings between control and sample.

DISCUSSION

These Peroxide Test procedures make possible not only the evaluation of the peroxides in petrolatums, but also the comparison of those petrolatums which, with the known methods of testing, would appear to have similar stability and quality.

Various types of petrolatum were tested accordingly. Table I summarizes the comparison of the Peroxide Values of representative samples of three types of petrolatum heated at 250° F. for various time intervals with their respective Peroxide Tests. The Peroxide Value is a quantitative expression for the amount of hydrogen peroxide formed in a kilo of petrolatum heated to 250° F. The Peroxide Test, on the other hand, is a qualitative test in which hours of heating and detection of the degree of peroxides formed provide a means of establishing the stability of petrolatums.

The Peroxide Value reported for each sample represents the average of at least three separate determinations, which never varied more than ±3%.

The accuracy and reproducibility of the above-described colorimetric method have been studied by means of dilute solutions of hydrogen peroxide of known concentration and by means of pure preparations of succinyl peroxide. For example, the concentration of hydrogen peroxide solution as given by the standard potassium permanganate method was 0.0036 mole per L., while the colorimetric procedure gave 0.0037 mole per L. The succinyl peroxide was prepared by the method of Clover and Houghton (4) and was recrystallized twice from acetone. The calculated weight per cent of active oxygen in succinyl peroxide is 6.83%; obtained by the colorimetric procedure 6.92%.

The results obtained confirm and substantiate what has already been reported, namely, that heat will accelerate the breakdown of petrolatum with the formation of additional peroxides (2).

These suggested Peroxide Test methods offer a means of determining a new constant or property for petrolatums to be used in pharmaceutical and cosmetic preparations. In the pharmaceutical use of petrolatum, it is of the utmost importance that careful consideration be given to the factor of stability. The values obtained by the above-described procedures offer themselves as a yardstick in this respect.

TABLE I.—COMPARISON OF PEROXIDE VALUES WITH PEROXIDE TESTS OF VARIOUS TYPES OF U. S. P. PETROLATUMS HEATED AT 250° F. FOR VARIOUS TIME INTERVALS

Type	Peroxide Values, Gm. of Hydrogen Peroxide per Kilo of Heated Petrolatum				Peroxide Test, Hr.
	0 Hr.	2 Hr.	3 Hr.	4 Hr.	
Amber	2.7×10^{-3}	2.8×10^{-3}	4.5×10^{-3}	5.5×10^{-3}	3
Snow White	1.7×10^{-3}	2.4×10^{-3}	5.7×10^{-3}	6.2×10^{-3}	2
Lily White	1.6×10^{-3}	1.7×10^{-3}	1.4×10^{-2}	1.7×10^{-2}	3

Petrolatums used in the preparation of pharmaceuticals and cosmetics should resist deterioration and be stable for a reasonable length of time in respect to odor, color, and the development of peroxides.

SUMMARY

A qualitative and quantitative Peroxide Test method is proposed for the determination of peroxides formed in petrolatums. These methods are simple and accurate.

A Peroxide Test of at least two hours is her with suggested as one of the minimum standar for petrolatums used in the manufacture pharmaceuticals and cosmetics.

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The Microbiological Determination of Choline*

By F. J. BANDELIN

The microbiological method for the determination of choline utilizing the growth response of the cholineless mutant of the mold, *Neurospora crassa*, is more sensitive and more specific than the chemical methods generally employed. It is not, however, as sensitive as microbiological methods for many other biologically essential substances such as the vitamins. Methionine, which interferes only slightly in the determination, may be removed by adsorption. The reagents, method, and preparation of samples are given, along with data obtained from determinations on various biological products generally used in the pharmaceutical industry. Reproducibility of results and recovery of added choline indicate that the method is reliable and satisfactory for these materials.

CHOLINE HAS been known as a chemical entity since Strecker first isolated it from hog bile in 1849 (1). Not until recently, however, has its important function as a lipotropic agent in nutritional physiology become known.

It is ubiquitous in nature, occurring in living tissues, both animal and vegetable, largely in the form of phospholipids; to a lesser extent as free choline; and in minute quantities as acetylcholine. In view of its new-found importance and wide distribution, it is natural that methods of isolation and determination be investigated in order to evaluate the natural sources of choline.

Chemical methods investigated to date are neither as highly sensitive nor are they as specific as for other biologically essential substances such as the vitamins. The microbiological method based upon the use of a cholineless m tant of the mold *Neurospora crassa* developed l Horowitz and Beadle (2) is considerably mo sensitive and more specific than any chemic method yet reported. The mutated strain produced by ultraviolet irradiation of the wi *Neurospora crassa* which when so treated los its ability to synthesize choline and consequentl requires exogenous choline for growth. Tl mutant therefore is designated as cholineles strain No. 34486. Growth of the cholinele *Neurospora* is stimulated not only by choline bu also by the following choline analogs: acety choline, dimethylaminoethanol, monomethy aminoethanol, phosphorylcholine, arsenocholin and by *dl*-methionine. Fortunately, with tl possible exception of *dl*-methionine, the concer trations of these compounds in biological materi are insignificant as compared with choline an hence do not interfere. Even methionine only 0.002 as active as choline (2) on a mol basis and may, in most instances, be ignored i choline assays since the ratio of choline to methic nine in most substances is such that any growt stimulation of the *Neurospora* due to methionin is within the experimental error inherent in th method. Sarcosine, betaine, creatine, ethanol amine, dimethylamine, trimethylamine, tetra methylammonium hydroxide and chloride do no interfere. Lecithin may serve as a source o choline for the *Neurospora* but the growth re sponse is slow, usually not more than 40 per cen of the total choline being accounted for in thre days' incubation. This is no doubt due to th poor solubility and slow hydrolysis of lecithin.

* Received Jan. 17, 1949, from the Research Laboratories, Flint, Eaton and Company, Decatur, Ill.

Where the concentration of methionine and certain nonbasic substances is such that it interferes in the determination, they may be removed through base exchange by passing the solution through a Zeolite column. The eluate is used for the determination.

PROCEDURE

By the following method it is possible to determine from 2 to 20 γ of choline in biological materials with a considerable degree of accuracy and reproducibility. Results of determinations made upon various pharmaceutical products and certain raw materials used in pharmaceutical products are given.

Reagents:

- 3% Sulfuric Acid
- Saturated Solution Barium Hydroxide
- 1 *N* SodiumHydroxide
- 0.3% Sodium Chloride Solution
- 5.0% Sodium Chloride Solution
- Standard Solution No. 1: Contains 1.0 γ of choline chloride per ml.
- Standard Solution No. 2: Contains 10 γ of choline chloride per ml.
- Basal Medium (Gm. per 500 cc.)

Ammonium Tartrate	5.0 Gm.
Ammonium Nitrate	1.0 Gm.
Potassium Phosphate (monobasic)	1.0 Gm.
Magnesium Sulfate (heptahydrate)	0.5 Gm.
Sodium Chloride	0.1 Gm.
Calcium Chloride	0.1 Gm.
Sucrose	20.0 Gm.
Biotin	5 γ
Asparagine	1.0 Gm.
Glutamic Acid	1.0 Gm.
Trace Salts Solution[1]	1 cc.

Adjust the *p*H to 5.0 and dilute to 500 cc.

Stock cultures of the cholineless *Neurospora crassa* are maintained on agar slants prepared from the basal medium by the addition of:

Agar	1.5%
Difco Yeast Extract	0.2%
Malt Extract	0.2%
Choline Chloride	1 γ per ml.

The slants are prepared in cotton plugged bacteriological tubes using 6 ml. of the above agar mixture and slanting to cool after autoclaving at 15 pounds pressure for ten minutes.

PREPARATION OF THE SAMPLE

A sample of from 50 to 500 mg. is weighed into a 50-ml. Erlenmeyer flask. Ten milliliters of 3% sulfuric acid is added; the flask is plugged with cotton and autoclaved at 15 lb. pressure for two hours.[2] After cooling, the material is quantitatively transferred, with the aid of 5 ml. of water, to a 50-ml. centrifuge tube and neutralized to Congo Red by the dropwise addition of saturated barium hydroxide solution. The barium sulfate formed by this step, together with any undissolved residue, is centrifuged down and the supernatant is filtered through a No. 50 Whatman paper. Three milliliters of distilled water is added to the residue in the tube and stirred with a glass rod. The tube is immersed in a boiling water bath for ten minutes with occasional stirring. The contents are cooled by holding the tube under the tap and then they are centrifuged. This washing is added through the filter to the previous supernatant. The clear filtrate is neutralized to litmus with 1 *N* sodium hydroxide and made up to volume with distilled water in a volumetric flask. This solution or subsequent dilutions should be prepared, based on the estimated choline content, so that the final dilution contains from 2.0 to 5.0 γ per ml. calculated as choline chloride.

If base exchange is necessary for the removal of interfering substances, 5 ml. of the neutralized solution is passed slowly through a Decalso column of the type generally used in vitamin separations. If the solution contains less than 1 γ of choline per ml., 10 ml. is passed through the column. It is then washed with two 5-ml. portions of 0.3% sodium chloride solution. A graduated test tube or cylinder is placed under the column and the choline is eluted with 10 ml. of hot 5% sodium chloride solution. The eluate, when cool, is adjusted to exactly 10 ml. with distilled water. Two or more samples should be run simultaneously at the same level.

ASSAY PROCEDURE

Ten-milliliter portions of basal medium are pipetted into 125-ml. flasks. The final volume, in each case, is adjusted to 20 ml. by the addition of test solution and water. For each series of determinations a standard series is set up containing 0.5, 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 40, 50, and 100 γ of choline chloride per flask. This is accomplished by using 0.5, 1, 2, 3, 4, 5, 7, and 10 ml. of standard solution No. 1 containing 1 γ of choline chloride per ml. and then using 1.5, 2.0, 2.5, 3.0, 4, 5, and 10 ml. of standard solution No. 2 containing 10 γ of choline chloride per milliliter. Test solutions may be run at several levels if desired. A convenient dilution of the unknown is one containing, in each ml., from 2 to 5 γ of choline calculated as the chloride.

After addition of the choline solutions to the flasks, the volume is adjusted to 20 ml. with distilled water. The flasks are then plugged with cotton and autoclaved at 15 lb. pressure for ten minutes. After cooling to room temperature, each flask is inoculated with one drop of a suspension of the *Neurospora conidia* in sterile water. This suspension is prepared by scraping some of the mold from the slant with a sterile wire loop and transferring it with sterile technique to a test tube containing from 5 to 10 ml. of sterile water. The tube is well shaken to disperse the *conidia* after which the suspension is withdrawn with a sterile pipette or dropper and one drop used to inoculate each of the flasks of media and test solution.

The flasks are incubated at 30° for seventy-two hours and the resulting orange-colored mold pads

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[1] Trace Salts Solution:

	Mg. per L.
Boron (as sodium borate)	0.01
[illegible]	0.02
[illegible]	0.2
[illegible]	0.1
[illegible]	0.02
[illegible]	2.0

[2] Refluxing with 5% sulfuric acid for five hours also apparently liberates all bound choline.

harvested by filtering each through a tared, sintered, glass crucible of medium porosity. The pads in the crucibles are washed with 10 ml. of distilled water and dried at 80°. After cooling in a desiccator the crucibles containing the mold pads are weighed and the weight of the mold pads determined. Choline values are calculated as the chloride in the usual way from a plot of the standard series.

A typical curve obtained by plotting a standard series is given in Fig. 1.

The detailed results obtained from determinations on a number of pharmaceutical biological products run at several levels, as well as recovery of added choline chloride, are given in Table I.

Results obtained on various dilutions of the different materials, both with and without base exchange, are given in Table II.

DISCUSSION

The growth response of the cholineless *Neurospora* is a linear function of the concentration over a rather narrow range. The optimum working range seems to be from 2 to 20 γ. The test solution should be prepared to have a concentration of from 2 to 5 γ of choline, as the chloride, per ml. where this is possible. Because of the difference in response of the *Neurospora* from time to time, standard

TABLE I.—RESULTS OF DETERMINATIONS ON A NUMBER OF PHARMACEUTICAL BIOLOGICAL PRODUCTS AND RECOVERY OF CHOLINE CHLORIDE

Material	Filtrate Used, Ml.	Choline Chloride Added, γ	Dry Weight of Mold, Mg.	Choline Found (as Chloride) γ per Flask	Choline Found (as Chloride) Mg. per Gm. of Sample	Choline Chloride Recovered γ
Liver Extract Injectable, 2 U. S. P. Units	1.0	0	19, 20	2.5	12.5	...
	2.0	0	43, 41.5	5.2	13.0	...
	3.0	0	50.5, 49	7.7	12.8	...
	1.0	5.0	50, 50	7.8	...	5.3
	2.0	5.0	55, 56	10.0	...	4.8
Liver B, Vitamins Injectable	1.0	0	35, 37	4.2	14.5	...
	2.0	0	53, 51	8.8	15.4	...
	3.0	0	60, 62.5	13.1	15.3	...
	1.0	5.0	54, 54	9.0	...	4.8
	2.0	5.0	62, 63.5	14.0	...	5.2
Liver Extract Injectable, 5 U. S. P. Units	1.0	0	15, 16	2.8	0.24	...
	2.0	0	31, 34	4.8	0.24	...
	3.0	0	47.5, 46	7.5	0.28	...
	1.0	5.0	45, 44	7.3	...	4.5
	2.0	5.0	57, 59	11.0	...	6.2
Whole Dried Liver Powder No. 1	1.0	0	48, 45	4.7	14.1	...
	2.0	0	62, 62.5, 60	10.4	15.6	...
	3.0	0	67, 70, 69.5	16.5	16.5	...
	1.0	5.0	61, 60, 64.5	9.6	...	4.9
	2.0	2.0	64, 65.5	12.3	...	1.9
Liver, Desiccated, Defatted	1.0	0	37, 37, 39	3.4	6.8	...
	2.0	0	54, 53, 55.5	7.0	7.0	...
	3.0	0	60, 62.5, 62	10.0	6.7	...
	1.0	2.0	51, 53, 50	5.8	...	2.4
	2.0	5.0	64, 62, 64	11.6	...	4.6
Yeast Autolysate No. 1	1.0	0	32, 33, 29	4.7	9.4	...
	2.0	0	52, 53.5, 53	9.0	9.0	...
	3.0	0	66, 68.5, 63	14.1	9.4	...
	1.0	5	54, 57, 59	10.1	...	5.4
	2.0	5	63, 67.5, 67	14.0	...	5.0
Yeast Autolysate No. 2	1.0	0	35, 34, 34	4.5	2.2	...
	2.0	0	66, 64.5, 64	8.8	2.2	...
	3.0	0	75, 78	11.4	1.8	...
	1.0	5	72, 72, 74	11.0	...	5.5
	2.0	5	79, 84, 81	14.5	...	5.7
Rice Bran Concentrate	1.0	0	20.5, 19, 22	2.3	11.5	...
	2.0	0	39.5, 36, 34	4.4	11.2	...
	3.0	0	50, 46.5, 47.5	7.0	11.6	...
	1.0	5	48, 53, 53	7.6	...	5.3
	2.0	5	59, 60.5, 57	10.8	...	6.4
Dried Yeast	1.0	0	19.5, 16, 19	1.8	3.6	...
	2.0	0	33, 31, 34.5	3.8	3.8	...
	3.0	0	46, 45, 51	7.0	4.6	...
	1.0	5.0	45, 45, 48.5	6.6	...	4.8
	2.0	5.0	57, 50, 55	8.6	...	5.0
Liver Concentrate Paste	1.0	0	37, 38, 36.5	3.2	3.2	...
	2.0	0	52, 49, 55.5	7.4	3.7	...
	3.0	0	59, 63, 60.5	10.8	3.6	...
	1.0	5.0	57, 57, 62.5	8.4	...	5.2
	2.0	5.0	64, 67, 61.5	12.7	...	5.4

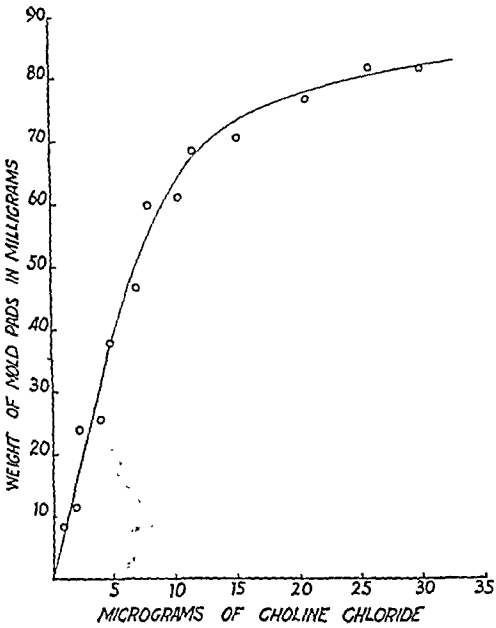

Fig. 1.

curves are not superimposable and a new standard curve must be prepared for each group of analyses. Duplicate or triplicate samples should be run on all standard and unknown solutions and an average mold weight figure should be used in constructing the standard curve or for computing the unknowns. Unknown samples may be run at several levels of choline concentration as a check. At higher concentrations, results are less accurate and may vary considerably due to linear deviation with a marked tendency toward high results. Where determinations are being carried out constantly with continued propagation of the mold, rapid or marked changes in the sensitivity to the mold are seldom noted. However, if the mold is carried on a slant and stored in a refrigerator for any length of time, it is well to carry out an orientation determination using graded amounts of choline standard solution to ascertain the intensity of the mold response to be expected and the level at which dilutions of unknowns might best be run. In a like manner, orientation determinations on unknowns should also be carried out at several levels in order to determine roughly the expected choline content and the proper dilution which will produce a concentration within the range of linear sensitivity.

The method requires rather large amounts of choline if compared in sensitivity to microbiological methods for other substances such as the vitamins. This, however, is in accord with the fact that the choline concentrations of tissues generally are of a magnitude greater than that of the vitamins. Similarly, the choline requirements of most species are far above the vitamin requirements.

The reproducibility of the method is fair when considered in the light of other microbiological procedures. Recovery of added choline is likewise satisfactory when considered statistically. Dry weights of molds from duplicate or triplicate flasks generally agree within 8% on the average with single wide variations observed from time to time. The recovery of added choline is usually within 10%. This, too, is an average figure.

TABLE II.—EFFECT OF BASE EXCHANGE ON CHOLINE VALUES FOUND

Sample	Micrograms of Choline Chloride Found without Adsorption	Micrograms of Choline Chloride Found after Adsorption
Liver Extract Injectable, 2 U. S. P. Units	5.5	5.1
	5.7	5.4
	5.6	5.1
Liver B Vitamins Injectable	4.4	4.0
	4.6	4.4
	4.9	5.0
Whole Dried Liver Powder	5.8	4.2
	6.1	4.0
	5.4	3.7
Liver Desiccated and Defatted	7.4	5.9
	7.2	5.6
	7.6	6.6
Yeast Autolysate No. 1	9.7	8.9
	9.4	8.3
	10.8	9.0
Yeast Autolysate No. 2	8.5	8.8
	9.4	8.6
	9.7	8.8
Rice Bran Concentrate	7.6	7.7
	8.4	7.5
	8.0	7.8
Dried Yeast	6.8	6.6
	6.9	6.4
	6.3	5.9
Liver Concentrate Paste	7.6	6.9
	7.3	6.3
	7.8	6.6

SUMMARY

Recent interest in choline as a therapeutic agent has created a need for methods of determination applicable to biological materials used in the pharmaceutical industry. The microbiological method based upon the growth of the cholineless mutant of the mold *Neurospora crassa* is considerably more sensitive and more specific than any of the chemical methods investigated to date. Although growth of the *Neurospora* is supported by various choline analogs, so that the reaction is not as specific as might be desired, these analogs, so far as is known, do not occur in any appreciable quantity in most biological substances. The *Neurospora* also responds to methionine but this response is slight and is not a serious deterrent in the method. Results obtained indicate that the method, while not as sensitive as many microbiological procedures, is reliable and reproducible and that added choline can be recovered with a fair degree of accuracy and consistency.

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Studies on Pharmaceutical Powders and the State of Subdivision. II. Surface Area Measurements of Some Pharmaceutical Powders by the Low-Temperature Nitrogen Adsorption Isotherm Technique*,†

By J. V. SWINTOSKY,‡ S. RIEGELMAN,§ T. HIGUCHI, and L. W. BUSSE

The practicability of the nitrogen adsorption method in determining the specific surface area of pharmaceutical powders has been illustrated in a previous paper. In this report the application of this technique to a number of nonrefractory pharmaceutical powders not previously investigated is presented. Comparisons are made of the specific surface areas of differently prepared forms of the same drug powders in order to provide a basis for interpreting the pharmacological, bacteriological, and pharmaceutical behaviors of these different forms. Other powders were investigated to extend the available quantitative data pertaining to the particle size and surface areas of some common pharmaceutical materials. The usefulness, characteristics, and limitations of this method in analysis of drug powders are discussed. Data are presented for measurements on bismuth subnitrate, bismuth subcarbonate, mercuric sulfide, and sulfanilamide. Also these studies include three different forms of sulfur, two forms of ammoniated mercury, two forms of sulfadiazine, and two forms of sulfathiazole.

THIS PAPER constitutes the second in a contemplated series of reports pertaining to studies on pharmaceutical powders and the state of subdivision. The first paper (1) described the apparatus and the method of approach followed in determining the specific surface areas of powders. In this report the application of the nitrogen adsorption (BET) method (2) to a number of pharmaceutical powders not previously investigated is presented.

The nitrogen adsorption method, because of its nature, has been applied only to a very limited extent to a few organic compounds and other nonrefractory substances. A brief summary of some of the characteristics (3) of this analytical technique serves to indicate its usefulness and limitations as applied to the analysis of pharmaceutical powders.

The method can be used for measuring the absolute surface areas of either porous or nonporous powders. Further, it can yield correct values for the surface average particle size if the material is nonporous, or if only micropores are present and their surfaces are not included in the surface area measurements. The method is restricted to solids with sufficiently large absolute areas to yield an adsorption value wel excess of the experimental errors involv Emmett (3) suggests that a 50-cc. sample powder should have a minimum absolute s face area of two square meters if the exp mental error is not to distort the true spec surface area.

In surface area studies of refractory substan it is customary to degas the samples at tempe tures of several hundred degrees in order to move all capillary condensed vapors and ga With nonrefractory pharmaceutical powd such elevated temperatures are impossible wi out altering the chemical and physical states these substances. Therefore the process necessarily restricted to much lower temperatu usually not exceeding 110°. It is unlikely t all capillaries which may be present are co pletely degassed at these temperatures; howev such capillaries are of little consequence pharmacy and therapeutics, especially since t play a very minor role in determining the solul ity and the rate of dissolution of ordinary cr talline materials. Indeed it would appear t surface area measurements which do not accou for the capillary surfaces should correlate bet with observed therapeutic and pharmaceu effects than measurements which include th microcapillary pores.

EXPERIMENTAL

In the present investigation several typical ph maceutical powders were studied. These w

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† This paper is based in part on a thesis submitted by Joseph V. Swintosky to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ American Foundation for Pharmaceutical Education Fellow, 1947–1948.

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chosen to permit comparison of the specific surface areas of differently prepared forms of the same drugs so that the data obtained could be used in interpreting the pharmacological, bacteriological, and pharmaceutical behaviors of these different forms. Other additional powders were chosen to extend the available data pertaining to the particle size and surface areas of some common pharmaceutical powders since such quantitative data are not generally available. The choice of the powders was necessarily restricted to those which could be measured by the nitrogen adsorption method. Substances such as boric acid, for example, which are volatile and decompose even at low temperatures, could not be studied by this technique.

Eight different types of drug powders were investigated. However, since several forms of some of these drug powders were studied, a total of thirteen samples was analyzed.

EXPERIMENTAL PROCEDURE AND DATA

Bismuth Subnitrate.—Mallinckrodt U. S. P.,[1] grade bismuth subnitrate was used without pretreatment. The powder was dried over granular anhydrous magnesium perchlorate in a desiccator for at least a week, as were all other powders unless otherwise indicated, prior to adsorption measurements.

Bismuth Subcarbonate.—A U. S. P. grade of bismuth subcarbonate was used without pretreatment.

Sulfanilamide.—Merck U. S. P. grade sulfanilamide was used without pretreatment.

Sulfadiazine.—Two different samples of sulfadiazine were analyzed:

(1) Calco U. S. P. grade sulfadiazine, supplied by Smith, Kline, and French Laboratories, was used without pretreatment.

(2) A microcrystalline sulfadiazine containing approximately 2% gelatin was used without pretreatment. This material likewise was obtained from the Smith, Kline, and French Laboratories.

Sulfathiazole.—Two different samples of sulfathiazole were analyzed:

(1) Smith, Kline, and French microcrystalline sulfathiazole containing approximately 2% gelatin was used without pretreatment.

(2) International Pulverizing Corp. "micronized" sulfathiazole was used without pretreatment.

Mercuric Sulfide.—Mallinckrodt red mercury sulfide (cinnabar) was used without pretreatment.

Sulfur.—Three different samples of sulfur were analyzed:

(1) Mallinckrodt U. S. P. grade precipitated sulfur was used without pretreatment.

(2) A colloidal sulfur was prepared by reacting sodium thiosulfate with hydrochloric acid in the presence of methyl cellulose.[2] The sulfur was separated from the water-soluble components by centrifuging in a Sharples centrifuge at 50,000 r. p. m. After drying the sulfur over magnesium perchlorate in a desiccator for two weeks, the mass of sulfur was crushed with a mortar and pestle to a granular consistency prior to measurement.

(3) A Doak Co., Inc., colloidal grade sulfur was also studied. This sulfur initially was approximately 40% pure, the remainder being proteinaceous material. The powder was placed in water allowing the proteinaceous material to be dissolved, and the suspended sulfur was then conveniently removed by centrifuging at 50,000 r. p. m. in a Sharples centrifuge. The sulfur was dried over magnesium perchlorate in a desiccator for a period of two weeks and the resulting mass was crushed to a granular consistency with a mortar and pestle prior to measurement.

Ammoniated Mercury.—Two samples of ammoniated mercury were analyzed:

(1) Mallinckrodt U. S. P. grade ammoniated mercury was used without pretreatment.

(2) A colloidal ammoniated mercury was prepared by reacting solutions of mercuric chloride and ammonium hydroxide in the presence of methylcellulose. The soluble components were separated from the ammoniated mercury by centrifuging with a Sharples centrifuge. After drying the mass of ammoniated mercury over magnesium perchlorate for two weeks, the material was crushed with a mortar and pestle and then analyzed in a nitrogen adsorption apparatus.

Nitrogen adsorption isotherms for these powders were obtained with a conventional BET apparatus as described in a preceding paper (1) of this series. Temperatures and evacuation times used in these analyses are listed for each powder in the tabulated data. As can be seen in the table, degassing temperatures in all cases were below 110°. At these lower temperatures it was necessary to carry out the evacuation process for relatively longer periods of time. Even then, as previously mentioned, the process may not have been complete. However, experience in this and other laboratories has shown that the values obtained by use of these lower degassing temperatures do not differ significantly from those obtained through use of much higher temperatures when the powders are relatively nonporous.

The relative vapor pressures of the adsorbent samples ultimately determined the temperatures used during the evacuation procedures, and the pumping time was in turn influenced by the temperature of the sample during the degassing period. All deadspace measurements were made with helium gas prior to the adsorption measurements.

RESULTS AND DISCUSSION

The results of the analysis on the various powders are shown in Table I and Figs. 1–12. Adsorption hysteresis was not observed in any of these measurements, so that the powders analyzed were assumed to be nonmicroporous in nature.

The samples of bismuth subnitrate and bismuth subcarbonate showed an appreciable difference in surface area and particle size. The larger particle size of bismuth subnitrate is evident qualitatively by feeling of the two powders or by observation with a microscope. The measurements would indicate that the bismuth subnitrate is about ten times as coarse as the bismuth subcarbonate.

Sulfanilamide and its derivatives showed marked differences in particle size. U. S. P. sulfadiazine was shown to have a specific surface area about five

[1] U. S. P. grade chemicals imply U. S. P. XII or XIII.
[2] Details regarding the preparation and utilization of several of these powders in bacteriostatic studies will be reported in a forthcoming paper of this series.

TABLE I.—SURFACE AREA MEASUREMENTS OF PHARMACEUTICAL POWDERS BY LOW TEMPERATURE NITROGEN ADSORPTION METHOD

Material	Weight of Sample, Gm.	Sample Number	Analysis Number	V_m, Cc.	Area, m²/Gm.	Average Area, m²/Gm.	Density	Surface Average Diameter of Particle, μ
Bismuth Subnitrate (U. S. P.)	17.08	4A	1	0.136	0.59_2	0.63	4.93	1.93
	17.90		2	0.154	0.67_0			
	17.90		3	0.145	0.63_1			
Bismuth Subcarbonate (U. S. P.)	18.24	5A	1	1.272	5.53	5.56	6.86	0.157
	14.54		2	1.282	5.58			
Sulfanilamide (U. S. P.)	29.79	6A	1	0.0609	0.26_5	0.28	1.4_9[a]	14.4
	29.79		2	0.0604	0.26_3			
	31.95		3	0.0684	0.29_8			
	31.95		4	0.0641	0.27_9			
Sulfadiazine (U. S. P.)	19.22	7A	1	0.360	1.57	1.57	1.50_0[a]	2.55
	19.22		2	0.360	1.57			
Sulfadiazine (microcrystalline, S. K. F.)	1.594	8A	1	3.46	15.1	15.1	1.50_0[a]	0.265
	1.520		2	3.47	15.1			
	1.528		3	3.47	15.1			
Sulfathiazole (microcrystalline, S. K. F.)	6.240	9A	1	0.936	4.07	4.12	1.50_0[a]	0.971
	5.980		2	0.960	4.18			
Sulfathiazole (Micronized, I. P. C.)	14.88	10A	1	0.473	2.06	2.10	1.50_0[a]	1.90
	14.88		2	0.492	2.14			
Mercuric Sulfide	12.45	11A	1	0.0865	0.32_4	0.33	8.10	2.24
	38.26		2	0.0752	0.32_7			
Sulfur (U. S. P.)	20.04	12A	1	0.0594	0.25_8	0.26	2.07	11.1
	20.97		2	0.0732	0.31_8			
	20.97		3	0.0531	0.23_1			
	21.94		4	0.0538	0.23_4			
	21.94		5	0.0612	0.26_6			
Sulfur (Doak Co., Inc.)	17.47	13A	1	0.911	3.96	3.96	2.07	0.732
	17.47		2	0.911	3.96			
Sulfur (chemically pptd. and spray dried)	4.290	14A	1	0.403	1.75	1.73	2.07	1.68
	12.91		2	0.392	1.71			
Ammoniated Mercury (U. S. P.)	48.70	15A	1	0.214	0.93	0.96	5.2_7[a]	1.19
	43.74		2	0.228	0.99			
Ammoniated Mercury (chemically pptd. and spray dried)	11.56	16A	1	0.934	4.06	4.06	5.2_7[a]	0.280
	11.56		2	0.934	4.06			

[a] Densities of these compounds were obtained with the nitrogen adsorption apparatus by determining the deadspace volume of a given empty sample tube followed by a determination of the deadspace of the same sample tube containing a known weight of powder. This then indicated the volume occupied by the known weight of powder. Dividing the weight of the sample by its volume gave the density directly.

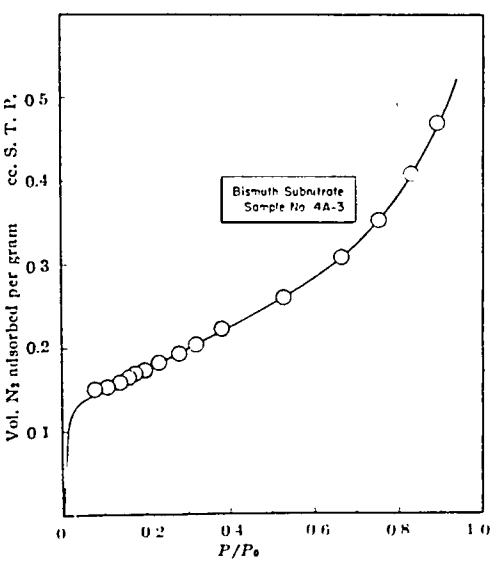

Fig. 1.—Adsorption of nitrogen on bismuth subnitrate at liquid nitrogen temperature.

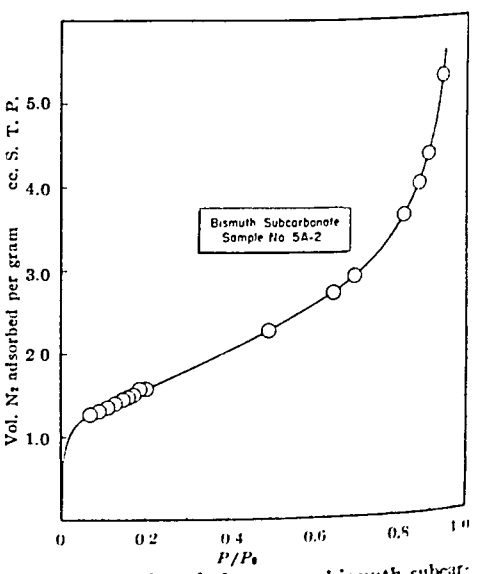

Fig. 2.—Adsorption of nitrogen on bismuth subcarbonate at liquid nitrogen temperature.

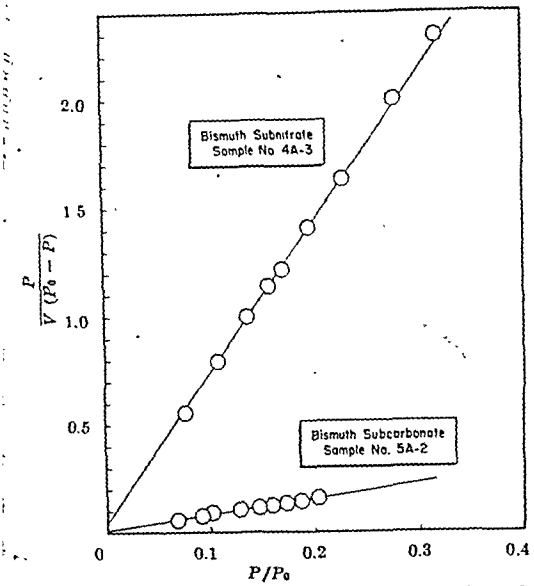

Fig. 3.—Adsorption data for nitrogen on bismuth subnitrate and bismuth subcarbonate, plotted according to the BET Equation.

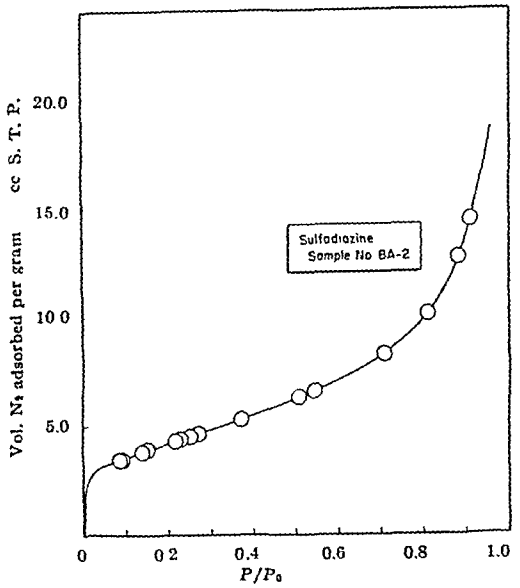

Fig. 5.—Adsorption of nitrogen on sulfadiazine at liquid nitrogen temperature.

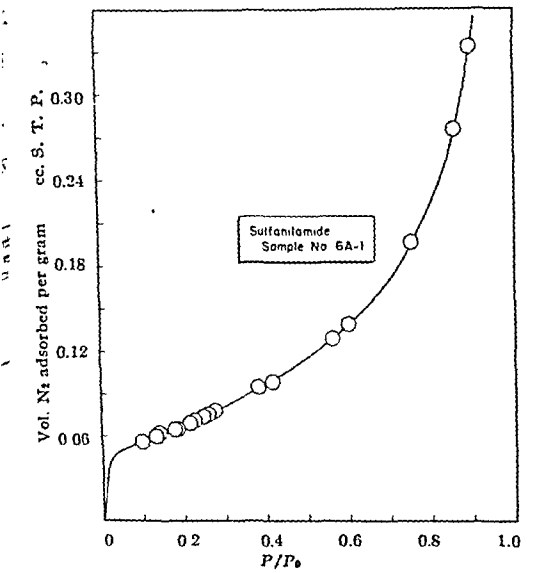

Fig. 4.—Adsorption of nitrogen on sulfanilamide at liquid nitrogen temperature.

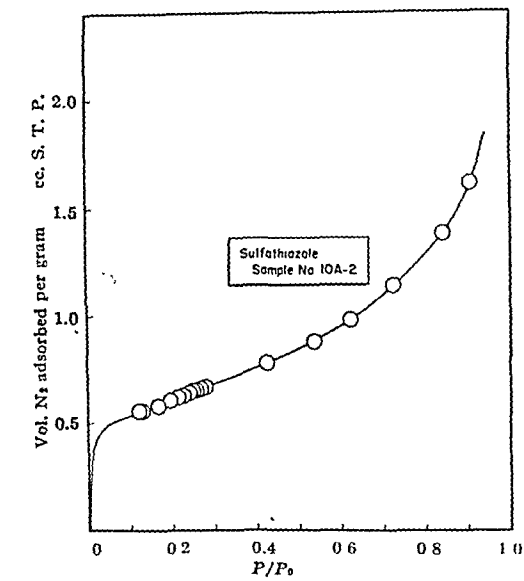

Fig. 6.—Adsorption of nitrogen on sulfathiazole at liquid nitrogen temperature.

times as great as the U. S. P. sulfanilamide, whereas the microcrystalline derivatives were considerably finer than either of the U. S. P. powders. The increased surface area of the microcrystalline derivatives is in keeping with the knowledge of increased rates of solution and absorption, and higher blood levels achieved with these substances in clinical usage (4, 5).

In the case of the sulfur powders, the U. S. P. sulfur was also shown to be extremely coarse; and it was difficult to measure accurately because of the larger relative magnitude of the deadspace error. The colloidal sulfur samples showed relatively large specific surface area values.[3] According to

[3] In the course of these measurements on the sulfur [illegible] observed that on immersion of [illegible] gen, the sulfur changed rathe[illegible] a white-colored powder. It is p[illegible] e change in color may occur with most colored compounds being cooled to this temperature since the concentration of the excited electronic states necessary for color production would be reduced and consequently would affect the light absorption properties of the material. The mercuric sulfide likewise displayed a color change from red to orange on cooling, and, like the sulfur, also returned to its original color after being removed from the liquid nitrogen bath.

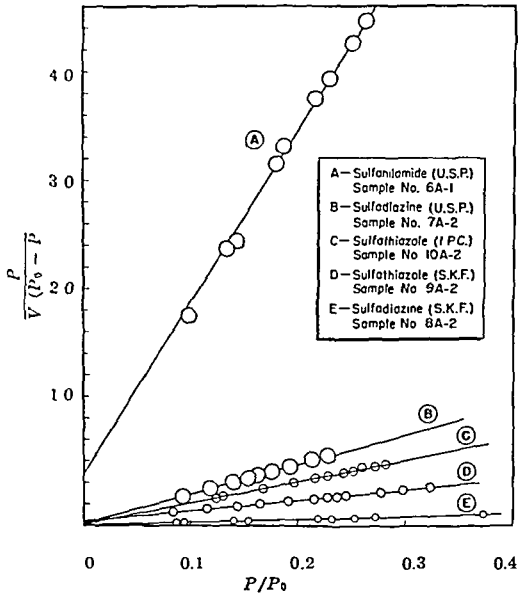

Fig. 7.—Adsorption data for nitrogen on sulfanilamide and on several grades of sulfadiazine and sulfathiazole, plotted according to the BET equation.

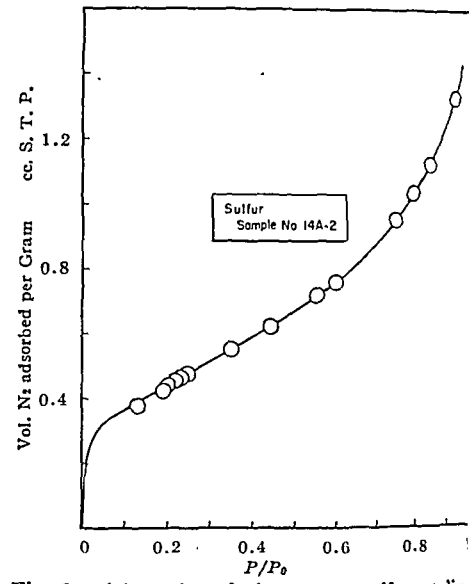

Fig. 9.—Adsorption of nitrogen on sulfur at liq nitrogen temperature.

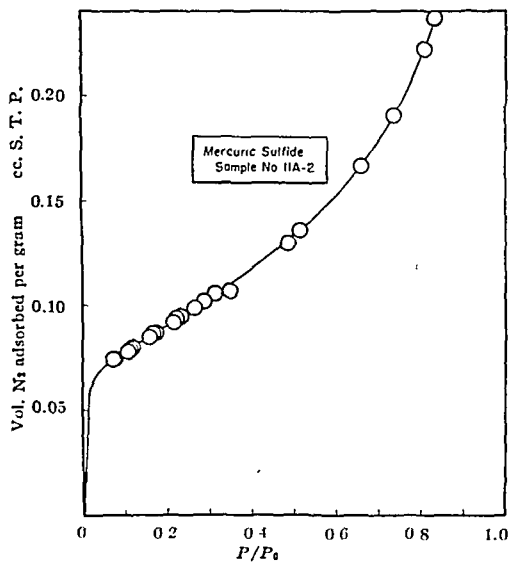

Fig. 8.—Adsorption of nitrogen on mercuric sulfide at liquid nitrogen temperature.

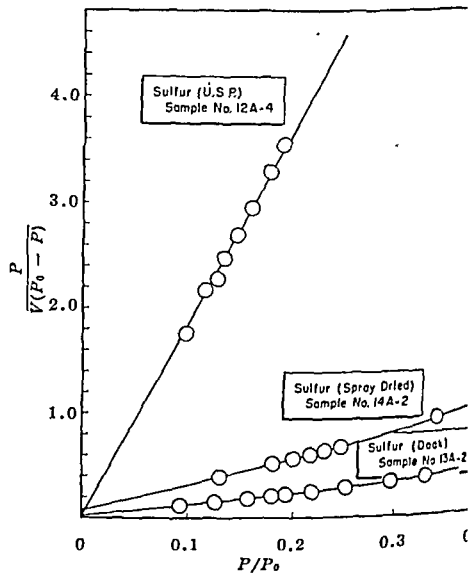

Fig. 10.—Adsorption data for nitrogen on seve grades of sulfur, plotted according to the BI equation.

the hypothesis that medicinal powders with higher specific surface areas display greater solution rates and greater activities, one might assume that the efficacy of the colloidal sulfur preparations would be proportionately greater than the noncolloidal forms.

No difficulties were encountered in the measurements of the specific surface areas of the ammoniated mercury samples. The specially prepared colloidal material was shown to have a specific surface ar about four times as great as the U. S. P. product.

It is apparent from the preceding data that t particle sizes and specific surface areas of differe pharmaceutical powders may vary over wide rang depending upon the composition of the materi and the method of manufacture. Since it appe that the efficacy of sparingly soluble drugs may va directly with the specific surface areas, it is evide

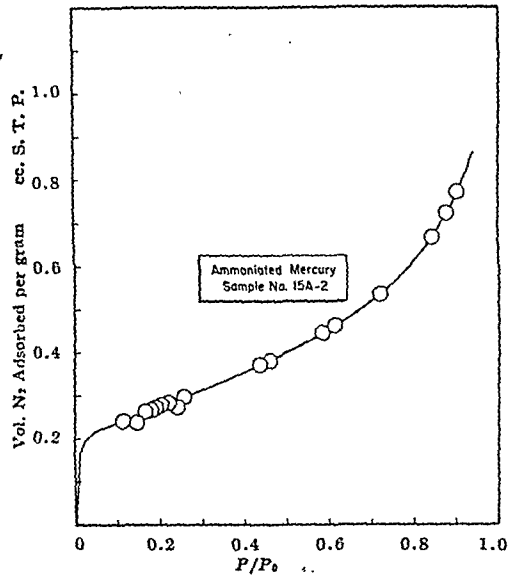

Fig. 11.—Adsorption of nitrogen on ammoniated mercury at liquid nitrogen temperature.

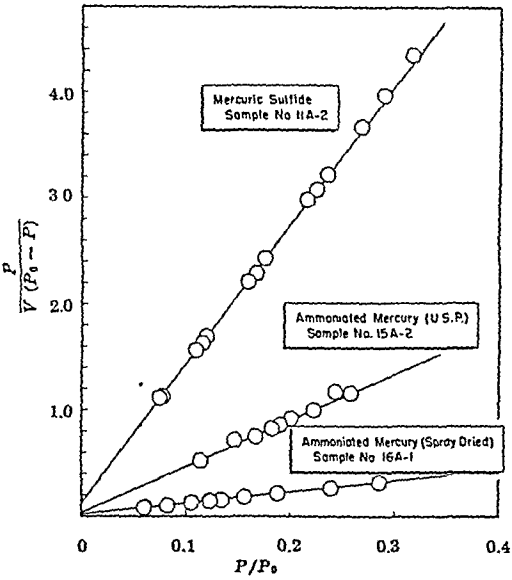

Fig. 12.—Adsorption data for nitrogen on mercuric sulfide and on two grades of ammoniated mercury, plotted according to the BET equation.

that further studies on these surface values should be made.

SUMMARY

Data are presented for specific surface area and surface average diameters for a number of pharmaceutical powders. Representative samples of these substances gave specific surface area values of 0.63 m.²/Gm. for bismuth subnitrate, 5.56 m.²/Gm. for bismuth subcarbonate, 0.28 m.²/Gm. for sulfanilamide, and 0.33 m.²/Gm. for mercuric sulfide. Three different forms of sulfur gave values of 0.26 m.²/Gm., 1.73 m.²/Gm., and 3.96 m.²/Gm., respectively. Two different forms of ammoniated mercury measured 0.96 m.²/Gm. and 4.06 m.²/Gm., respectively. Two different forms of sulfadiazine measured 1.57 m.²/Gm. and 15.1 m.²/Gm., and two different forms of sulfathiazole measured 2.10 m.²/Gm. and 4.12 m.²/Gm., respectively.

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Microscopic Characters of Some of the Crystalline Sympathomimetic Amines*

By GEORGE L. KEENAN†

MICROSCOPIC descriptions recorded in this report are the result of the study of samples of the crystalline sympathomimetic amines and their derivatives, principally furnished by Haley, and used by him in the chemical differentiation of these compounds. For convenience of reference, data on *l*-ephedrine hydrochloride and ephedrine sulfate, already on record in the literature (1) are included.

* Received September 19, 1948.
† Formerly Microanalyst, Food and Drug Administration. Present Address: Strongsville, Ohio.

The data here recorded are based on a determination of the optical crystallographic properties of the compounds, such data being available from a microscopic examination with the polarizing microscope and applying the immersion method (see Table I). Where possible, a similar microscopic study has been made of any crystalline addition products that could be formed by the use of the appropriate microchemical reagent. The compounds will be considered alphabetically for convenience of reference.

Refractive Indices.—α = 1.535 (commonly shown lengthwise on elongated fragments), β = indet. γ = 1.620 (shown crosswise on elongated fragments); both ±0.002.

Chloro-auric acid reagent also forms an addition product of yellow rods and needles with this compound. With crossed nicols the extinction is parallel and the sign of elongation with the selenite plate and crossed nicols is negative. In convergent polarized light (crossed nicols), no interference figures are observed since the crystals extinguish sharply. The significant refractive indices are α = 1.568 (commonly shown lengthwise on rods), β = indet., γ = >1.734 (crosswise on rods). β is probably shown on plates tipped on edge.

TABLE I.—TABULATION OF DATA

Substance	Habit	Extinction	Elongation	Refractive Indices α	β	γ
d-Desoxy ephedrine HCl	Fragments	...	...	1.530	1.537	1.615
dl-Desoxy ephedrine HCl	Plates	Parallel	Negative	1.535	Indet.	1.620
l-Ephedrine HCl	Rods	Parallel	Negative	1.530	1.603	1.638
dl-Ephedrine HCl	Fragments	...	...	1.570	1.608	1.630
Ephedrine SO_4	Plates	Parallel	...	1.540	1.565	1.587
Paredrine HBr	Fragments	...	...	1.560	1.680	1.734
Tuamine SO_4	Plates	...	...	1.458(ω)	...	1.468(ε)
Vonedrine HCl	Rods	Parallel	Negative	1.577	Indet.	1.603

d-DESOXYEPHEDRINE HYDROCHLORIDE

In Ordinary Light.—The habit is not significant, the material breaking up into irregular fragments when crushed for microscopic study.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—It is characteristic of the substance to show many fragments that do not extinguish sharply with crossed nicols.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The substance is biaxial and axial plane and optic axis figures are frequently observed. Optic sign (+). 2*E*, not large.

Refractive Indices.—α = 1.530, β = 1.537, γ = 1.615; all ±0.002. The β-value is frequently shown.

With chloro-auric acid, yellow rods and needles are formed, many of the rods having square ends. The rods and needles show parallel extinction with crossed nicols and with the selenite plate and crossed nicols the sign of elongation is negative. In view of the fact that the crystals extinguish sharply with crossed nicols, no interference figures are observed in convergent polarized light (crossed nicols). The significant refractive indices of this addition product are: α = 1.580 (shown lengthwise, and very common), β = indet., γ = slightly higher than the *n* for methylene iodide (1.734), but many rods match this liquid crosswise; both ±0.002.

dl-DESOXYEPHEDRINE HYDROCHLORIDE

In Ordinary Light.—The substance consists of very small, platy crystals, some of which are roughly six-sided in habit, and others elongated.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is usually parallel on elongated fragments and the sign of elongation with selenite plate and crossed nicols is negative. The rods and needles invariably extinguish sharply.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—No interference figures observed.

l-EPHEDRINE HYDROCHLORIDE

In Ordinary Light.—The habit is elongated prisms and rods. It probably crystallizes in the orthorhombic system.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is parallel and the sign of elongation with the selenite plate and crossed nicols is negative.

Refractive Indices.—α = 1.530, β = 1.603, γ = 1.638 (1).

dl-EPHEDRINE HYDROCHLORIDE

In Ordinary Light.—The habit is not significant the substance breaking up into irregular fragments.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—Most of the fragments extinguish sharply with crossed nicols and occasionally some remain bright under the same conditions.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The substance is biaxial and occasionally optic axis figures are observed. Optic sign (−).

Refractive Indices.—α = 1.570 (common), β = 1.608, γ = 1.630 (also common); all ±0.002.

EPHEDRINE SULFATE

In Ordinary Light.—The habit is hexagonal plates.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is parallel on elongated forms.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—No interference figures are shown. Optic sign (+). 2E is large.

Refractive Indices.—$\alpha = 1.540$, $\beta = 1.565$, $\gamma = 1.587$. Plates extinguishing sharply show index 1.540 in one extinction position and 1.565 in the other (1, 2).

PAREDRINE HYDROBROMIDE

In Ordinary Light.—The habit is largely irregular fragments without significant form.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—Some fragments extinguish sharply with crossed nicols while others remain bright when the microscope stage is revolved.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—Partial biaxial interference figures are frequently observed, these showing one optic axis in the microscopic field. Optic sign (−).

Refractive Indices.—$\alpha = 1.560$, $\beta = 1.680$, $\gamma = 1.734$; all ± 0.002.

A solution of paredrine hydrochloride becomes blood-red on the addition of chloro-auric acid reagent.

TUAMINE SULFATE

In Ordinary Light.—The substance consists of colorless, irregularly shaped plates and fibrous flakes.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The larger, irregular plates usually do not extinguish sharply when the microscope stage is revolved. Birefringence not strong but moderate.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The larger, irregular plates not extinguishing sharply show a distinct uniaxial interference figure with positive optic sign. These figures occur quite frequently.

Refractive Indices.—$\omega = 1.458$, $\epsilon = 1.468$; both ± 0.002.

The rods and needles formed with picric acid show inclined extinction and characteristic anomalous polarization colors with crossed nicols, the colors consisting of peculiar blues and purples.

VONEDRINE HYDROCHLORIDE

In Ordinary Light.—The substance consists of small, rod-like fragments.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is parallel and the sign of elongation is negative on the elongated fragments.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—No interference figures observed.

Refractive Indices.—$\alpha = 1.577$ (commonly shown lengthwise), β = indet., $\gamma = 1.603$ (commonly shown crosswise); both ± 0.002.

The chains of prisms which the compound forms with chloro-auric acid are yellow. The prisms vary in habit and are probably monoclinic. Prisms showing inclined extinction and the maximum double refraction have a minimum refractive index (α) of 1.603 and a maximum value (γ) of 1.734; both ± 0.002 (2).

SUMMARY

The principal microscopic characters of some of the crystalline sympathomimetic amines have been determined by the immersion method and suitable microchemical reagents, the optical crystallographic data being recorded in Table I.

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Effect of Metabolite Antagonists on Histidine Decarboxylase*

By J. M. BEILER, ROBERT BRENDEL, MIRIAM GRAFF, and GUSTAV, J. MARTIN

The results of a study of histidine decarboxylase metabolite antagonists are reported. The compounds used in the study included a series of vitamin antagonists, compounds containing phenolic hydroxyl groups and compounds structurally related to histidine.

SOME ten years ago Werle (1, 2) and Holtz (3) reported the existence in animal tissues of an enzyme capable of decarboxylating histidine to form histamine. This they called histidine decarboxylase. The decarboxylation was slow, and the amount of CO_2 formed was too small to be measured in the Warburg, so the reaction was studied pharmacologically.

EXPERIMENTAL

The procedure used was a modification of that of Werle and Herrmann (2). Two grams of guinea-pig kidney (fresh weight) were ground with sand in 4 cc. of distilled water. The mixture was centrifuged and 1 cc. of the supernatant was added to 2.5 cc. of

* Received Nov. 27, 1948, from the Research Laboratories The National Drug Co., Philadelphia, Pa.

M/15 phosphate buffer *p*H 8.5, containing 10 mg. of *l*-histidine. One and seven-tenths cubic centimeters of this solution was placed in the Warburg cups in which the experiment was run, and 0.3 cc. of a neutral solution of the substance whose effect was being determined was added to this. The mixture was shaken at 37° under N_2 for two to three hours, and the amount of histamine formed was determined by comparing the amplitude of contraction produced in the isolated guinea-pig intestine with that produced by known quantities of histamine. The amount of histamine formed in the reaction was quite constant, and varied from 3 to 4γ/cc.

Preliminary experiments had shown that the tissue extract alone, and the *l*-histidine alone, produced no contraction of the guinea-pig intestine, so that these were not run routinely as controls. Controls were run with tissue extract and histidine, and tissue extract without histidine plus the compound being tested. In cases where a stimulation or inhibition was obtained, an additional control was run with tissue extract plus the compound being tested plus a measured amount of histamine. This showed whether the effect noted was due to an action of the compound being tested on histidine decarboxylase or to an action on the response of the guinea-pig intestine to histamine.

RESULTS

The effects of three series of compounds on the action of histidine decarboxylase were studied.

(A) A series of vitamin antagonists was investigated. This was done on the premise that, should some vitamin function as the coenzyme of the decarboxylase, its displacement by a suitable antagonist should result in an inhibition of the enzyme.

(B) Werle (4) showed that the enzyme was inhibited by adrenaline, dopa, and several other compounds of like structure. This he ascribed to the presence in the various molecules of phenolic hydroxyl groups. Accordingly, a number of compounds containing phenolic hydroxyl groups were studied.

(C) A number of compounds whose structures possessed varying degrees of similarity to that of histidine were investigated. The object here was to determine whether an inhibition of the enzyme action could be achieved by a displacement of the substrate.

The results are listed in Tables I, II, and III. Inhibitions were calculated by comparing the amounts of histamine formed in the controls (histidine and tissue extract) with those formed in the presence of added amounts of the compounds being tested.

TABLE I.—EFFECT OF VITAMIN ANTAGONISTS ON THE ACTION OF HISTIDINE DECARBOXYLASE

Compound	Concentration (Mg./Cc.)	Inhibition, %
Glucoascorbic Acid	1.0	0
Isoriboflavin	1.0	0
Desthiobiotin	1.0	0
Pantoyl taurine	1.0	0
Pyrithiamine	1.0	Stimulation
7-Methyl Folic Acid	1.0	Stimulation
Desoxypyridoxine	1.0	0
Pyridine β-sulfonamide	1.0	0

TABLE II.—EFFECT OF PHENOLIC COMPOUNDS ON THE ACTION OF HISTIDINE DECARBOXYLASE

Compound	Inhibition, % 1.0 Mg./Cc.	0.1 Mg./Cc.	0.01 Mg./Cc.
Catechol	100	40	..
Dopa	100	25	..
N-methyl dopa	40	..	..
Salicylic Acid	20	..	..
Tyrosine	0	..	..
N-methyl tyrosine	0	..	..
Phenylalanine	0	..	..
p-Fluorophenylalanine	0	..	..
Benzoquinone	100	80	0
Inositol	0	..	..

TABLE III.—EFFECT OF COMPOUNDS STRUCTURALLY RELATED TO HISTIDINE ON THE ACTION OF HISTIDINE DECARBOXYLASE

Compound	Inhibition, % 2.5 Mg./Cc.	1.0 Mg./Cc.
Imidazoleamino methyl sulfonic acid	35	0
N-Sulfanilyl-4-aminobenzimidazole	..	20
Benzoxazolone	..	20
3-Benzothiophene α-amino propionic acid	..	25
β-2-thienyl alanine	..	40

DISCUSSION

The results show that none of the vitamin antagonists tested produced any inhibition of the action of histidine decarboxylase. Three explanations may be offered for this finding. First, the enzyme may be associated with a coenzyme which is analogous to none of the compounds tested. Second, there may be no coenzyme. Or, finally, the coenzyme may be so tightly bound to the enzyme that it cannot be displaced by the methods used. Either of the last two possibilities is consistent with the observation of Werle (5) that the enzyme does not lose activity on dialysis.

No explanation is readily forthcoming for the fact that both pyrithiamine and 7-methyl folic acid were found actually to stimulate to a slight extent the action of histidine decarboxylase. It is possible that there is some other enzyme present in the tissue extract which attacks histidine, and that this is inhibited by the vitamin antagonists in question. This would, in effect, raise the concentration of histidine available to the histidine decarboxylase, and thus increase the activity of the enzyme. Some support for this view is provided by Werle's observation (4) that thiamine actually inhibited the action of histidine decarboxylase. Folic acid, on the other hand, was tested and was found to be without effect.

The effect of phenols on the decarboxylase is striking; as may be seen from Table II, some of these were the most effective compounds tested. The extent of the inhibition caused by these phenolic compounds was dependent on the number of hydroxyl groups present, at least within the limits of the compounds tested. The effect seems to be peculiar to phenolic hydroxyls; inositol, a compound rich in nonphenolic hydroxyls, was completely inactive.

The extent of the inhibition varied with the number of phenolic hydroxyls present, at least within the limits of the compounds tried; all the compounds

with two hydroxyl groups were more active than those with only one. The effectiveness of the compounds varied inversely with the complexity of the side chain; catechol was more active than dopa, which in turn was much more active than N-methyl dopa; salicylic acid was more active than tyrosine. Whether the action of benzoquinone is analogous to that of the phenolic compounds is not known.

The third group tested comprised a number of compounds structurally related to histidine. The results indicate that there is no rigid specificity here; compounds of quite diverse structures were effective as inhibitors. The effect was small, being much less than that produced by some of the phenolic compounds, and it may be that this is a measure of the failure of any of the compounds tested to represent what would be a truly specific displacer structure.

SUMMARY

Three series of compounds have been tested to determine their effect on the action of histidine decarboxylase. These included vitamin antagonists, compounds containing phenolic hydroxyl groups, and compounds structurally related to histidine.

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The Oxytocic Evaluation of Posterior Pituitary Solutions, U. S. P., the Guinea-Pig Uterus*,†

By CLARENCE E. POWELL and EDWARD E. SWANSON

Several methods for evaluating the oxytocic potency in posterior pituitary solutions are discussed. Experiments were carried out in which the method for the blood pressure response in chickens was applied to the Official U. S. P. Guinea-Pig Uterus Method with certain changes.

THE PRESENT METHOD of evaluating the oxytocic potency in posterior pituitary solutions, as described in the U. S. P. XII and XIII, has many disadvantages. Morrell (1) enumerated the difficulties encountered with this procedure and suggested a method based on the quantal response of a single guinea-pig uterus, divided into 8 strips in a common bath with separate levers. With an all or none response, it was shown that the error of each individual test could be computed independently and the potency estimated in terms of a standard from regression lines.

Smith and Vos (2) have designed a method for the chicken's blood pressure response to pituitary preparations, first described by Coon (3). The potency and experimental error were computed mathematically. Later, Vos (4) proposed the same method of calculation for estimating the potency of ergonovine on the rabbit's uterus. By this method, the standard doses remained constant and the unknown doses varied in size, so that some gave shorter or equal latent periods and others, longer latent periods. Thompson (5) further investigated this design on the chicken's blood pressure response to pituitary solutions and reported results that were in close agreement with the official uterine method. This same design was also applied by Thompson to two tests on the official guinea-pig uterus method. His results indicated close agreement with the official assay.

Thompson, however, found a significant difference between the potency on the blood pressure of chickens and the potency on the guinea-pig uterus in one out of 17 parallel determinations, and suggested that the blood pressure method cannot always be relied upon to estimate the oxytocic activity. More recently, Holton (6) reported a statistical method of standardization of posterior pituitary extract on the isolated uterus of the rat.

In view of the advantages claimed for the chicken's blood pressure method in evaluating the oxytocic principle of posterior pituitary solutions, it appeared worth while to investigate further the guinea-pig uterus method. We have, therefore, applied the experimental design described by Vos to certain changes of the Official U. S. P. Guinea-Pig Uterus Method.

METHOD

The apparatus was the same as that used for the official method—namely, a constant temperature

* Received Dec. 31, 1948, from the Lilly Research Laboratories, Indianapolis 6, Ind.
† The authors are indebted to Lester Le Compte, Jr., for assistance in testing and computing the results of this in- [illegible] loyd Miller, chairman of the U. S. P. [illegible] for his valuable suggestions and

bath with a 100-cc. capacity muscle chamber. Recording of uterine movements was made in the usual manner with either a gravity or a frontal writing lever, having a ratio of 1:3. Van Dyke and Hasting's solution was used for a bathing fluid. In brief, this bathing solution was prepared as described by Morrell, Allmark and Bachinski (1), page 441, paragraphs 2, 3, and 4. With this procedure the necessary *p*H of 7.4 to 7.5 can be maintained throughout the test. Standard pituitary dilutions were made up in saline solution to contain 0.02 unit per 1 cc. The unknown dilutions were made up in various strengths with saline solution.

Nonpregnant guinea pigs weighing between 200 and 350 Gm. were used. The animal was killed by a blow on the head or decapitated and the entire uterus body immediately removed. The two horns were divided at the bifurcation and immersed in van Dyke–Hastings solution, contained in a shallow dish. If large, the horns may be divided longitudinally.

Before a trial dose of the standard pituitary solution was given, the uterus was acclimated to the bathing fluid for twenty to thirty minutes to insure complete relaxation. Two or three changes of the bathing fluid aided in adjustment of the uterus. When the uterus had relaxed to a constant base line a 0.02-unit dose of the standard solution was tried The bathing fluid was changed when the peak of the contraction was reached. When the muscle was completely relaxed, a second 0.02-unit dose was tried. Usually ten-minute intervals between doses were taken throughout the assay. If the two standard contractions were of the same height and appeared to be maximum, decreasingly smaller doses were tried until a definite submaximal dose had been established. This submaximal dose was maintained throughout the test.

Following the determination of the submaximal dose of the standard pituitary solution, an equal volume of the unknown was tried. The height of contraction of this dose determined the subsequent unknown volumes to be given. If, however, the unknown response was above the standard contraction, smaller volumes of the unknown were used until the contractions were equal to or below the standard contraction. Conversely, if the unknown response was below the standard, larger volumes of the unknown were given equal to and above the standard responses.

A total of 13 doses, 7 standards and 6 unknowns, were found to give reliable results. The final in all cases was the standard. When the test had been completed, the heights of contractions of the standard and unknown doses were measured to the nearest 0.5 mm. and the data treated as described by Vos. A typical calculation is shown in Table I. This procedure has been used in our laboratory for the past five years and compared with the official U. S. P. method.

The results found in fourteen assays out of more than one hundred are outlined in Table II.

TABLE I.—CALCULATION SHOWING RESPONSE OF GUINEA-PIG UTERUS TO POSTERIOR PITUITARY EXTRACT

Date: June 28, 1948
Compound: Pit. Ext.
Serial No.: 12
Animal or Muscle: Guinea-Pig Uterus

Dose No.	Standard Log Dose	Log Response	Interpolate	Unknown Log Dose	Log Response	x	y
1	0.602	0.839	...	...	...	...	...
2	...	...	0.839	0.602	0.833	0.000	0.006
3	0.602	0.839	...	...	...	...	...
4	...	...	0.857	0.544	0.851	0.058	0.006
5	0.602	0.875	...	...	...	...	...
6	...	...	0.884	0.602	0.887	0.000	−0.003
7	0.602	0.892	...	...	...	...	...
8	...	...	0.898	0.653	0.909	−0.051	−0.011
9	0.602	0.903	...	...	...	...	...
10	...	...	0.909	0.544	0.903	0.058	0.006
11	0.602	0.914	...	...	...	...	...
12	...	...	0.914	0.653	0.919	−0.051	−0.005
13	0.602	0.914	...	...	...	...	...

M = 0.003637 = 100.8%
Standard Error = 3.07%
Actual Potency = 100%

TABLE II.—RESULTS OF COMPARATIVE ASSAYS BETWEEN THE OFFICIAL U. S. P. METHOD AND THE VOS DESIGN

Sample No.	U. S. P. Assay, %	Vos Assay, % ± S. E.
1	100	104.10 ± 4.30
2	120	118.00 ± 14.86
3	100	85.61 ± 12.72
4	100	90.20 ± 6.56
5	100	94.01 ± 6.05
6	100	104.30 ± 10.77
7	100	93.26 ± 3.19
8	100	96.54 ± 3.97
9	80	80.74 ± 4.86
10	80	89.69 ± 5.53
11	80	83.63 ± 3.30
12	100	100.80 ± 3.07
13	110	112.80 ± 1.93
14	90	90.03 ± 6.63

SUMMARY

1. A method for the evaluation of the oxytocic principle in posterior pituitary preparations has been described.

2. The method requires a simple constant temperature bath and the use of uteri from guinea pigs weighing between 200 and 350 Gm.

3. The method utilizes the Vos design for conducting the test and for computation of potency and standard error.

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Fluorometric Determination of Ketotetrahydropyridines. An Improved Method for Urine*,†

By ELMER De RITTER, FRED W. JAHNS, and SAUL H. RUBIN

An ether extraction procedure has been developed for the fluorometric determination in urine of 3,3-diethyl-2,4-diketotetrahydropyridine. By increasing the salt content of the urine, to promote "salting out" of the drug, and by buffering with borate at *p*H 9–9.5, quantitative extraction is achieved, while considerably less nonspecific fluorescence is carried along than in the direct method previously reported. Sensitivity and precision are thus increased. The method has been applied successfully, with good reproducibility and good recoveries, to urines from normal subjects and from patients with renal and hepatic disease at excretion levels as low as 1 to 2 mg. per day.

THE USE of the drug, 3,3-diethyl-2,4-diketotetrahydropyridine, Presidon,[1] as a sedative-hypnotic (1–5) has stimulated work on assay methods. In an earlier paper from this laboratory (6), a method for urine assay was described, which was based on measurement of the fluorescence of a direct dilution in alkaline, alcoholic solution, before and after quenching with hydroxylamine. Though suitable for most clinical studies, this method was not as sensitive as desired because of the presence in urine of considerable, nonspecific fluorescent substances. Also, in occasional pathological urines, hydroxylamine has been found to quench appreciable amounts of interfering fluorescence so that high "apparent drug" values are obtained.

Although an adequate correction for the amount of nonspecific fluorescence quenched by hydroxylamine can normally be made by measuring excretion of "apparent drug" in a control period before administration of the drug, it has been found much simpler and more direct to eliminate much of this interference by extraction of Presidon from urine with diethyl ether. With this modification both the sensitivity and the precision of the method have been increased and the need for an internal standard eliminated.

* Received Jan. 24, 1949, from the Nutrition Laboratories.

with hepatic and renal dysfunction.

[1] This drug was formerly referred to as Persedon (5–7).

DESCRIPTION OF METHOD

Extraction.—Pour about 23 ml. of 25% aqueous potassium chloride into a 125-ml. separatory funnel. Pipette 1.00 ml. of urine, or an aliquot containing at least 5 γ of Presidon, and 1.00 ml. of a saturated solution of sodium tetraborate into the funnel. Extract with three successive 25-ml. portions of diethyl ether, shaking vigorously for two minutes for each extraction. If an emulsion forms, transfer the entire contents of the separatory funnel to a stoppered centrifuge tube, centrifuge briefly to break the emulsion; transfer quantitatively back to the separatory funnel for separation. Combine the three ether extracts in a 250-ml. Erlenmeyer flask, add 1.0 ml. of a 1% aqueous solution of ascorbic acid, and boil off the ether on a steam bath. To prevent bumping, add a small, alundum antibump stone or carry out the ether evaporation under a gentle stream of nitrogen. Remove the last traces of ether under a stream of nitrogen and avoid overheating of the residual aqueous solution. Dilute with water to a Presidon concentration of approximately 1 γ per ml., using at least 10 ml. of water.

Fluorometry.—Pipette 5.0 ml. of diluted extract and 10.0 ml. of ethanol into duplicate glass-stoppered tubes. To one of the tubes add 1.00 ml. of water and to the other 1.00 ml. of a 10% aqueous solution of hydroxylamine hydrochloride. Then add 1.00 ml. of 10% aqueous sodium hydroxide to both tubes. Set up a similar pair of tubes with 5.0 ml. of a standard solution containing 1.00 γ of Presidon per ml. in place of the 5.0 ml. of urine extract.

Stopper all tubes and mix well. Loosen but do not remove the stoppers of the tubes containing hydroxylamine and immerse these tubes in a wire rack in a constant-temperature bath at 75–80° for twenty minutes. At the end of the heating period, remove the tubes and cool to room temperature in a cold water bath.

Take fluorescence readings of the unheated and the hydroxylamine treated standard and unknown with the same filters as used for thiochrome measurements (the primary ultraviolet filter having a peak of 3700 Å. and the secondary filters a peak of 4600 Å.). Use a fluorescent glass block to set the fluorophotometer to a suitable galvanometer response just prior to each reading.

Calculation:

If S = fluorometric reading of untreated standard
S_B = fluorometric reading hydroxylamine-treated standard
U = fluorometric reading of untreated urine solution
U_B = fluorometric reading hydroxylamine-treated urine solution
A = ml. of urine extracted
D = ml. to which urine extract is diluted
V = total volume of 24-hour urine in ml.

Then

$$\frac{U - U_B}{S - SB} \times \frac{D \times V}{1000 \times A} =$$

milligrams of Presidon/day

EXPERIMENTAL

Recovery of Presidon.—The completeness of recovery by this procedure of Presidon extracted from aqueous solutions and from urines containing known added amounts of Presidon, is shown in Table I. In the case of the urines, corrections have been made for the slight quenching of nonspecific fluorescence by hydroxylamine as determined by parallel assay of urines to which no Presidon was added.

As a further check on the completeness of extraction of Presidon from urine, a series of urines with and without 25 γ of added Presidon per ml. were extracted and the fluorescence of the residual aqueous phases read in the usual manner after adding 10 ml. of ethanol, 1.00 ml. of 10% sodium hydroxide, and 1.00 ml. of water to 5.0 ml. of each solution. The comparative fluorometric readings are shown in Table II. The lack of significant differences between urines with and without added Presidon indicates that no Presidon remains in the aqueous phase after three ether extractions Unextracted urines under these conditions showed a difference of approximately 50 galvanometer divisions due to the added Presidon.

Comparisons with Direct Method.—Comparative assays of urines without Presidon were carried out by the present extraction procedure and the direct method (6) to determine the relative magnitude of the blank fluorescence readings and of the "apparent drug" excretions. Table III gives a comparison of fluorescence blanks obtained with both methods at the same over-all dilution, namely 1.00 ml. of urine to 170 ml. In each case the reagent blank was subtracted from the observed reading. These typical data show the effectiveness of ether extraction in eliminating practically all of the blank fluorescence.

The extent of the quenching of non-Presidon fluorescence by hydroxylamine is given in Table IV for both methods. It is obvious that a considerable reduction in the "apparent drug" values is achieved by the present method.

TABLE I.—RECOVERY OF PRESIDON AT VARIOUS CONCENTRATIONS

Presidon Concentration, γ/Ml.	Per Cent Recovery: Water Solution	Per Cent Recovery: Urine
1	95	95
5	93	94
10	96	101
25	100	99
50	99	98

TABLE II.—RESIDUAL FLUORESCENCE OF AQUEOUS PHASE AFTER EXTRACTION OF URINES WITH AND WITHOUT ADDED PRESIDON

Urine Sample	Fluorometric Reading: No Presidon	Fluorometric Reading: 25 γ of Presidon per Ml.
W. H.	16	15
H. M.	70	70
G. S.	14	13
M. D.	58	58
T. L.	92	97

TABLE III.—COMPARISON OF FLUOROMETRIC BLANKS BY DIRECT AND ETHER EXTRACTION METHODS

Urine Sample	Blank Fluorescence Reading: Direct Method (6)	Blank Fluorescence Reading: Ether Extraction Method
T. L.	31	0.5
M. D.	16	1.5
G. S.	6	0.5
H. M.	24	1.0
W. H.	5	0.0
L. C.	10	1.5
W. M.	4	0.0

TABLE IV.—QUENCHING OF NONSPECIFIC FLUORESCENCE BY HYDROXYLAMINE DIRECT VS. ETHER EXTRACTION METHOD

Subject	Type[a]	Apparent Presidon, Mg./Day: Direct Method (6)	Apparent Presidon, Mg./Day: Ether Extraction Method
L. D.	N	2.0	0.0
F. J.	N	1.2	0.2
J. B.	N	2.4	0.4
S. R.	N	1.1	0.0
G. S.	H	5.1	0.0
M. M.	H	26.8	2.7
W. H.	R	8.7	1.8
W. M.	R	9.9	0.6
M. M.	R	17.5	4.3
T. L.	R	18.7	0.0

[a] N = normal; H = hepatic dysfunction; R = renal dysfunction.

In contrast to the direct method, in which an internal standard is obligatory, the diluted ether extracts in the present method have been found to cause no quenching of Presidon fluorescence and, hence, do not require an internal standard. Presidon extracted from aqueous solution and from urines has been found to be stable in the ether solution for at least three hours at room temperature.

DISCUSSION

Since Kubli and Schmid (7) reported that Presidon exhibits an intense blue fluorescence in strongly alkaline solution under ultraviolet light, several fluorometric assay procedures have been devised (5–8). Kubli (8) suggested ether extraction of undiluted urine after addition of sodium bicarbonate to minimize extraction of interfering fluorescent compounds. In this laboratory, however, that procedure frequently caused persistent emulsions and yielded consistently low and variable Presidon recoveries. Wilbrandt and Jaeger (5) also were unable to achieve quantitative extraction of Presidon by repeated shaking out with ether from urine diluted with an equal volume of borate buffer (*p*H 9.2). They adopted the expedient of using a single shake-out with ether, which gave about 45% recovery in the ether extract under the specified conditions. Since a quantitative extraction is inherently more reliable than an equilibrium distribution, the present method was devised to achieve this result, while retaining the advantages of the ether extraction, such as greater sensitivity, decreased interference by nonspecific fluorescence, and elimination of the need for an internal standard. We have, however, adopted the borate buffer used by Wilbrandt and Jaeger, in preference to bicarbonate as used by Kubli and Schmid. The use of borate, in conjunction with heavy salt addition to the urine, has been effective in promoting quantitative extraction with ether without troublesome emulsions.

In extensive use of this procedure for assay of normal and pathological urines, emulsion formation was encountered in less than 20% of the urine samples. A brief centrifugation was sufficient to break such emulsions in all cases.

The selection of reagents, particularly ether, which will not cause excessive fluorescence blanks, is an important consideration. Mallinckrodt's Analytical Reagent grade of anhydrous ether has been found satisfactory. Denatured alcohol (2B) has been found to be equally as good as U. S. P. ethanol. If high reagent blanks are encountered, redistillation of solvents is necessary.

The addition of ascorbic acid to the ether extract before evaporation of the ether is recommended to prevent slight losses of Presidon, which occur in the absence of ascorbic acid. The destruction is probably oxidative, possibly due to traces of peroxides in the ether. In the earlier procedure from this laboratory (6), urine solutions were heated with and without hydroxylamine before fluorometry, but in the present procedure only the blank tubes containing hydroxylamine are heated in order to avoid the possibility of further oxidative losses of Presidon in the nontreated tubes. Since the amounts of extraneous fluorescence are usually small in the ether extracts, this has little effect on the "apparent drug" values.

Satisfactory recoveries of as little as 1 γ of Presidon added to 5 ml. of urine (corresponding to a daily excretion of 0.2 to 0.4 mg. per day) have been achieved by the present method. The sensitivity and precision obtainable in an excretion study, however, are more dependent on the day-to-day variations in the "apparent drug" excretions. Since these values are relatively small, as shown by the typical data in Table IV, and the average variation from day to day is usually less than 0.4 mg., it is possible to measure Presidon excretions of 1 to 2 mg. per day with good precision.

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Determination of Heavy Metals in Somewhat Colored Solutions*,†

By E. A. McARTHUR

A new method is described for determining heavy metals. It embraces the compensation of color in the solution by multiple compartment observation tubes. Some applications, precautions, and limitations are discussed.

SINCE the official methods for the estimation of heavy metals are dependent upon the evaluation of the amount of colored sulfide formed, any color inherent in the solution of the unknown itself presents a rather difficult problem in the determination.

The British Pharmacopoeia has attempted to circumvent this difficulty by addition of dilutions of caramel to the lead standard, thus equalizing the color shown by the sample. This treatment has met with only limited success inasmuch as the color of a given unknown may or may not be of a comparable quality with that of caramel.

Introduction of a smaller amount of the sample to the lead standard has also been resorted to by

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† Presented August 3, 1948, before the Canadian Foundation for Advancement of Pharmacy, at Windsor, Ontario, Canada.

the British Pharmacopœia, the estimation of the heavy metals content being based on the difference in the quantity of substance represented in the test and that in the standard. Rosin (1) likewise made use of this technique in his text. This approach does not actually compensate for the color of a given sample. It appears rather to be an attempt to unify the chemical constituents of the standard and unknown solutions to act as a background for the development of the test.

Examination of the constituents of the ash from an organic compound has been resorted to in some instances by the United States Pharmacopœia where color interferes in the application of the official method. Here again the procedure is lengthy and results may be seriously misleading. Ignition temperatures certainly would volatilize mercury compounds; lead, copper, and some other metals no doubt would be lost in varying degrees depending upon the intensity of heat applied in incineration.

The method herewith presented embraces the compensation of color in the unknown by utilization of dual compartment observation tubes. Thus, the specific color of the sample under test is represented with the standards in making the measurement.

METHOD

Two observation units, each consisting of two Nessler tubes of 50 ml. and 100 ml. capacity, are used. These are designated as A (50 ml. tube = A_1 and 100 ml. tube = A_2) and B (50 ml. tube = B_1 and 100 ml. tube = B_2) in Fig. 1. The 50-ml. tube is suspended in its respective 100-ml. tube by a firm fitting but adjustable rubber collar of about 1/4-inch thickness cut from a rubber stopper of No. 7 or No. 8 size. This rubber ring or collar in normal practice will be near the top of the smaller tube and, when the unit is assembled, will rest upon the top of the larger tube, thus forming a dependable support for the former. For ease of manipulation, these rings are wetted with water just prior to the beginning of a determination. The entire length of walls of both large tubes (A_2 and B_2) should be given an opaque covering, preferably white paper, to prevent interference by light from outside sources.

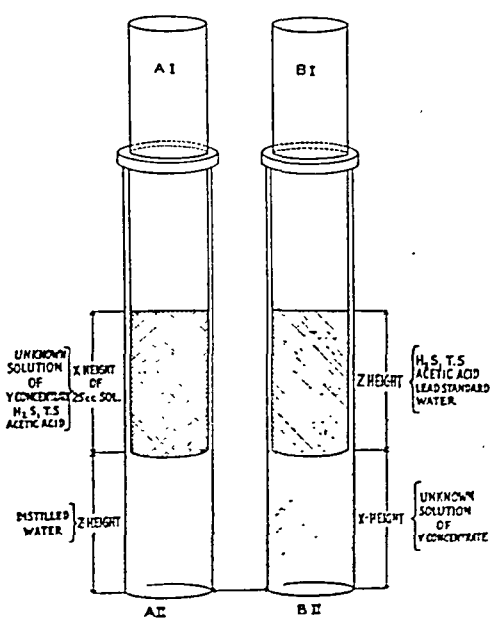

Fig. 1.—Observation units.

The unknown is processed in the observation unit A while the standard is developed in unit B. Prepare 75 ml. of solution of the unknown of such a concentration that 25 ml. will contain the quantity of sample desired for the test. Into tube A_1 introduce 25 ml. of this solution and then add the remainder to tube B_2 until a depth of about 1/4 inch greater than in A_1 has been reached. To tube A_2 add distilled water to a level identical with that of the liquid in B_2. Lower tube A_1 into A_2 by adjusting the rubber collar on A_1 so that the end of the smaller tube is just submerged in the distilled water contained in the larger tube. Now add the volume of lead standard desired to tube B_1 and sufficient distilled water to make a total of 25 ml.; insert this tube then into position in B_2. Acidify the contents of tubes A_1 and B_1 each with 1 ml. of acetic acid and mix contents thoroughly. Adjust the superimposed tube B_1 until its lower end is immersed in the liquid in B_2 and so that the color when viewed from above exactly matches that in the A unit observed under the same technique. (It may be necessary to tilt each unit sidewise, to free any entrapped air beneath the smaller tube, before making the above equalization.) Quickly introduce into each of A_1 and B_1, 10 ml. of hydrogen sulfide T.S. and mix the contents with glass rods. Allow to stand for ten minutes, at the end of which time comparisons may be made. These directions are specifically intended for evaluating the unknown in terms of "less than" or "greater than" a definite set limit.

If it is desired that the specific heavy metal content be ascertained more closely, a series of knowns can conveniently be prepared in a set of tubes to be used alternatively in the B_1 position, these usually increasing from one tube to the next in lead content by 5 μg. Each of these must be matched in unit B with the unknown in unit A, as outlined above, before addition of the hydrogen sulfide T.S. Interpolation of values between the knowns yields results within the precision of the heavy metals determination by official methods.

In those instances necessitating the processing of the unknown in an alkaline solution the procedure has likewise been successful, 5 drops of a 10% aqueous solution of sodium sulfide being substituted for the hydrogen sulfide T.S.

Some chemical items requiring alkali to produce solution may be neutralized and then acidified with acetic acid without crystallizing or precipitating. Thus, the original directions may be followed therefrom.

EXPERIMENTAL

In order to establish the validity of this procedure, a number of dyes were selected to act as color back-

grounds. Each was first subjected to the conditions of the test in aqueous concentrations of 1–500,000 and 1–1,000,000. Comparisons were then made against their respective controls of like concentrations but with 10 ml. of distilled water replacing the 10 ml. of hydrogen sulfide T.S. Three of these dyes were found to be completely resistant to the reducing action of the hydrogen sulfide for a period of one hour and were consequently chosen for performance runs in the determination. The dyes F.D. & C. Yellow No. 5—Tartrazine, F.D. & C. Red No. 2—Amaranth and F.D. & C. Blue No. 1—Brilliant Blue approximate the primary colors.

When the above outlined method was applied, using each of the dyes in an aqueous concentration of 1–1,000,000, a standard of 5 μg of lead was definitely discernible, in all cases, from its respective color background. In fact, in the instances of the red and the blue shades, the color difference from unknown to standard seemed to be sharpened or enhanced over that witnessed for the general official heavy metals test. This latter phenomenon was interpreted as being due to the component colors (dye and metallic sulfide) having the effect of producing a new shade rather than just a one-color development as in the usual heavy metals estimation.

Using the same dye concentrations as mentioned immediately above, studies were made to ascertain whether color development, due to metallic sulfide formation, followed the same trend in both units. As directed under the method, each observation unit was processed to that point where the colors were equalized. A standard solution containing 10 μg of lead was added to both A_1 and B_1 tubes, the contents mixed, hydrogen sulfide T.S. added, again mixed, and allowed to stand during ten minutes reaction time. Nearly all such studies resulted in identical color matches in the two units. Occasionally a variation could be noted and these were usually interpolated to be of the order of 2 μg of lead. On repeating such an estimation, it was not always possible to show this variation and it was concluded that under the conditions of evaluation, no doubt this represented the limit of sensitivity to be expected especially by the naked eye. Higher lead concentrations were not attempted in this study, since unknowns of a comparable level would allow dilution, thus requiring less sample and consequently resulting in less color.

Assuming that the sensitivity of the test is in the order of that shown in the experiment described above, in an assay representing one Gm. of substance in the 25 ml. of unknown solution, it is feasible to determine as low a metal contamination as 5 p. p. m. as lead. This experience duplicates the performance of the official procedure, the latter being free from the complications of color in the solutions under examination.

DISCUSSION

Besides the general rules of colorimetry, certain precautions must naturally be observed in the procedure. The rubber support rings for the inserted or suspended tubes must be maintained in their originally adjusted positions to insure correct results. Occasionally an unknown is discovered to be of such a chemical nature as to produce a foreign color when brought in contact with hydrogen sulfide. Often this foreign color does not develop in the alkaline medium with sodium sulfide. As was indicated in reference to the dyes used for the experimental background, some colored solutions undergo reduction in presence of hydrogen sulfide with a resulting diminution of shade or the development of an entirely different color. In such an instance, reduction of the color by other chemical means prior to submission to test should be attempted. Alert observation by the chemist should make it possible to detect either of the above-mentioned difficulties.

Perhaps the most outstanding disadvantage of the proposed method is the size of sample required. This is of quite definite significance when analyzing materials from research origin. Many of the items which today require such examination are found to be extremely costly. To this end, the possibility of semimicro application should not be overlooked. In some laboratories such a method may appear disadvantageous in that it seemingly does not lend itself to instrumentation.

Limitations of the method, in addition to those of the official procedure, are:

1. Unknown solution must not possess a color intensity such that prevents interpretation of that formed by the metallic sulfides.
2. The medium resulting from dissolving the unknown must not be of such high concentration or specific nature as to inhibit the development of the colored sulfides.
3. As pointed out above, sample must not be chromatically reactable with the sulfide.

It is felt that in the hands of the experienced analyst the method serves a very definite purpose. In this laboratory it has been used with a great deal of success for the determination of heavy metals in such items as sulfonamides, antibiotics, vitamins, quaternary ammonium compounds, and in the control of a number of organic intermediates in the manufacturing processes. It is particularly adaptable for direct application to many of the official items such as Gelatin, U. S. P., and Dextrose, U. S. P., which now require ashing, semiashing or some other form of pretreatment. Other naturally colored items such as Pectin, N. F., and Whisky, N. F., present color difficulties in conducting the official heavy metals test which can be overcome by the proposed method. While the method described herein is potentially adaptable to practically all official items now specifically examined for heavy metals, it is recognized that a considerable amount of additional study on individual items is necessary before suggesting this as an official procedure but the method is recommended as an improvement over the present official heavy metals test in those instances where interfering colors must be dealt with.

SUMMARY

A method is offered for the direct determination of heavy metals in substances producing somewhat colored solutions.

The suggested procedure is intended primarily as a limit test but has been used successfully for quantitative estimation of heavy metals content.

A proposal is made for the investigation of the outlined method in application to official items producing interfering colors in solution, and especially those requiring special pretreatment.

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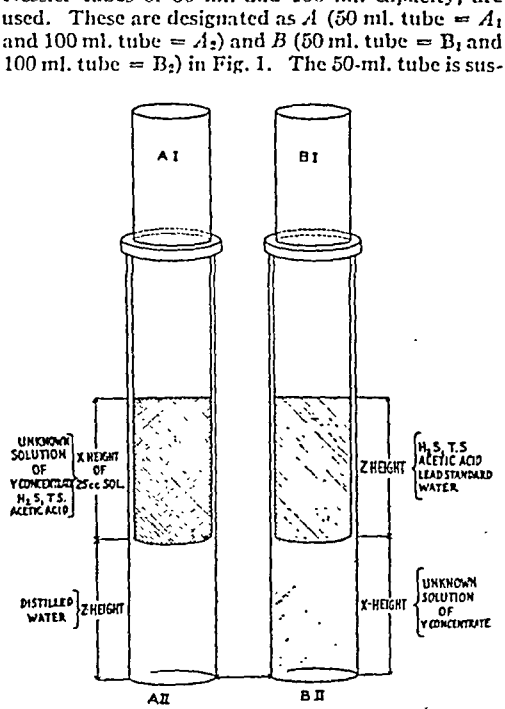

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A proposal is made for the investigation of the outlined method in application to official items producing interfering colors in solution, and especially those requiring special pretreatment.

The principles of the test can be expanded to a number of measurements in general colorimetry.

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Fluorescence of Powdered Vegetable Drugs with Particular Reference to Development of a System of Identification*

By CHARLES R. CHASE, JR.† and ROBERTSON PRATT

An introductory section includes a brief résumé of previous observations on the fluorescence of crude drugs and drug products exposed to ultraviolet radiation. A key for identification of powdered vegetable drugs based on their fluorescence under ultraviolet radiation is presented. Possible application of the method and its shortcomings are discussed.

FLUORESCENCE excited by visible light is reported to have been observed first in 1602 by Cascariola, a cobbler living in Bologna, Italy (1). According to DeMent the first demonstration of fluorescence excited by invisible rays was provided by Sir George Stokes about the middle of the last century. He observed that a tube containing a solution of quinine bisulfate appeared bluish when held in appropriate regions of the violet band of the visible spectrum and that the blue color became more intense as the tube was moved progressively toward the shorter wave lengths and even when it was moved some distance into the dark region beyond the visible violet rays.

Applications.—During the nearly one hundred years that have elapsed since this observation, much important information about fluorescence has been acquired and has been applied in the development of modern concepts in certain phases of physics and chemistry. Practical applications have been made in analytical chemistry and in industry. However, despite the fact that the first observation of fluorescence in ultraviolet radiations was made on a plant product that is an important drug, i.e., quinine, and despite the fact that several investigators have reported differences in fluorescence of several crude drugs or their preparations under ultraviolet radiatio pharmacognocists have made comparatively li use of the phenomenon. The only practi applications in common use appear to be in detection of contamination by rodents (1*a*) the well-known fluorescence test for distingui ing rhapontic rhubarb from the official U. S. species and the N. F. VIII identity test for *drastis*.

Heimstädt (2) described a fluorescence mic scope in 1912 and suggested its practical ap cation in pharmacognosy for the detection small amounts of ergot in meal. The follow year Wasicky (3) in extending these observati directed attention to the high degree of sensitiv of the fluorescence method of detecting the pr ence of certain drugs or their constituents. showed, for example, that quinine could be tected under the fluorescence microscope ev when diluted 1:10⁵. He further applied t technique to the detection of dandelion in chico of rumex in gentian, and to differentiation various fibers.

There seems to have been little impetus given to this line of investigation in crude drugs, however, until 1929 when Göllner (4) reported on the difference in behavior of three groups of drugs which he classified as responsive, indifferent, or phosphorescent in ultraviolet radiations. Following this observation a number of workers reported on the fluorescence of crude drugs (either powdered or granulated), of a number of galenical preparations, or of isolated and purified constituents of the drugs (5–14). Most of these, however, were comparatively limited studies, directed toward detection of adulterants in crude drugs (7–10), the separation of two or three related crude drugs (7, 12), a mere cataloging of the behavior of official preparations of different pharmacopœias under ultraviolet radiations (5, 6, 10) or of the behavior of extracted active principles (11).

* 1948, from the College of Pharmacy, San Francisco, Calif.
ntific Section, A. PH. A., San Francisco meeting, August, 1948.
† Recipient of the Kilmer Prize for 1948.

Leupin and Steiner (13) observed that some drugs which exhibited a similar fluorescence might be made to fluoresce differently by treatment with appropriate reagents, and they suggested that this differential behavior might have valuable applications in the identification of crude drugs; but a search of the literature has failed to reveal any large-scale systematic attempts to work out a satisfactory system for identifying crude drugs by means of their behavior when exposed to ultraviolet radiation.

The work reported in this paper was undertaken to study the possibility of developing a system of identification of powdered vegetable drugs based on their fluorescence characteristics. It is conceivable that such a system might possess distinct advantages over, or might serve as a useful adjunct to, the present methods that depend upon microscopic examination of histological features and on microchemical tests, both of which are quite time-consuming. Saving of time and resolution of doubtful specimens might be expected to be among the advantages of a system of identification based on fluorescence. Such a system has been worked out tentatively and is the subject of the present report. Further experimentation is required to develop satisfactorily all of the details but it is believed that the work is sufficiently advanced and is of sufficient interest to warrant presentation in its present form. The only major piece of apparatus that is required, i.e., a suitable source of ultraviolet radiations and appropriate filter, is relatively inexpensive and is common in most laboratories equipped for physical measurements.

EXPERIMENTAL

Materials and Methods.—The fluorescence of 151 identified powdered drugs under ultraviolet light was studied. The source of radiation was a mercury arc provided with a Corning ultra-type filter which transmits the near ultraviolet (above 3100 Angstrom units) and absorbs the wave lengths in the visible portions of the spectrum. It may be assumed that the 3650 band of the mercury arc was primarily responsible for the fluorescence that was observed in this work.

Since it was soon found that direct observation of the dry powders was unsatisfactory, several methods of mounting were investigated. Finally, the following three methods were adopted.

Method A.—A small amount of the powdered drug was suspended in a solution of nitrocellulose in amylacetate[1] on a microscope slide. The solvent evaporated and the slide was ready for use in a few minutes. The mounting fluid does not fluoresce when exposed to the wave lengths that are transmitted by the Corning filter that was used. Slides prepared by this method may be kept for several months without apparent change of fluorescent characteristics.

Method B.—A small amount of the powdered drug was placed on a slide and treated with 1 *N* NaOH in methanol and permitted to dry for approximately thirty minutes. Then the mounting fluid consisting of nitrocellulose in amylacetate was added as in Method A and the slide was ready for use in a few minutes. The fluorescence characteristics of slides prepared in this way gradually change over a period of several weeks.

Method C.—A small amount of the powdered drug placed on a microscope slide was treated with 1 *N* NaOH in methanol and was observed under the ultraviolet radiations while still wet. Observations are made most easily in a dark room but satisfactory results can be obtained merely working in a shaded corner of the laboratory if the slides are placed on a black background and are shielded from direct visible wave lengths.

RESULTS

When mounted as in Method A, 51 of the drugs (33.7% of those studied) exhibited fluorescence. All colors of the spectrum were represented and there appeared to be some correlation between the part of the plant used and the color of fluorescence. Of the drugs that fluoresced red, 95.5% were flowers, fruits, or leafy portions of plants; of those that fluoresced yellow, 85.8% were underground portions of plants; and of those that were blue, 71.5% were seeds. It is worthy of comment that all powdered seeds that exhibited fluorescence in this method of mounting emitted a blue color. In some cases drugs that fluoresced did not appear as a uniform color throughout, but had a spotted appearance. The spots were almost always yellow or green. The fluorescent spots frequently appeared in drugs, the main fluorescence of which was some other color so that the spots appeared as yellow or green flecks against a colored background. In other cases they constituted the only fluorescence of the powdered drug so that they were seen against a dark gray or black background.

The drugs that exhibited fluorescence were reasonably well segregated into groups, but more than 60% of the drugs were in the large group that did not fluoresce at all. It was in an attempt to break down this nonfluorescing group that mounting Methods B and C which involve pretreatment with NaOH were developed. This search proved fruitful since 93.4% of the drugs that were studied were found to fluoresce in one or more of the methods and it was found that the fluorescence of a given drug was generally different in the different methods. This seems to indicate that the fluorescence of the drugs was due to a number of different compounds and that several such compounds probably occur in a single species.

The exact color of fluorescence of each drug in each method of mounting was listed using Ridgeway's "Color Standards and Nomenclature" (15) as a guide. The extremely large number of shades of each color listed in this work makes matching any sample with a definite shade in the charts a comparatively simple matter. Occasionally it was found that more than one drug fluoresced the same color. In such cases, once the color had been satisfactorily determined, it was necessary to estimate the intensity of fluorescence. Ultimately this should be worked

[1] This is sold as Testor's Model Airplane Cement.

out quantitatively with reliable instruments. For the present purpose, however, simple visual observation and grading upon an arbitrary scale of intensity ranging from 1 to 4 was adequate. By comparison of the color and intensity of fluorescence of a drug mounted in each of the three ways cited, it is generally possible to identify it quickly. In practical identification of an unknown, the powdered drug is first classified according to its color or lack of color in Method A; then it is further classified according to its color or lack of color in Methods B and C, and, if necessary, according to the estimated intensity of fluorescence in Method C. For convenience and for permanent reference in the present investigation a color rating based on Ridgeway's standards (*loc. cit.*) was assigned to the fluorescence of each drug when mounted in each of the three ways cited above. An individual with a normal sense of color, however, can satisfactorily separate the drugs into comparatively small groups and in many instances can make a final identification without recourse to such an extensive color guide. These observations have led to the development of the following key for identification.

Key for Identification of Powdered Vegetable Drugs on Basis of Their Fluorescence in Ultraviolet Radiations

I. Colored in Method A
- A. Blue in Method A
  - 1. Colored in Method C
    - (*a*) Colorless in Method B
      - (1) Blue in Method C
        - Almond meal
        - Delphinium
    - (*b*) Blue in Method B
      - (1) Blue in Method C
        - Corydalis
        - Gelsemium
    - (*c*) Blue-green in Method B
      - (1) Blue in Method C
        - Spigelia
        - Veratrum viride
      - (2) Blue-green in Method C
        - Senega
        - Black mustard
      - (3) Green in Method C
        - Yellow mustard
  - 2. Colorless in Method C
    - (*a*) Colorless in Method B
      - Chondrus
    - (*b*) Blue in Method B
      - Linum
      - Squill
    - (*c*) Blue-green in Method B
      - Kava-Kava
    - (*d*) Green in Method B
      - Staphisagria
    - (*e*) Yellow-green in Method B
      - Ignatis
      - Nux Vomica
    - (*f*) Blue spots in Method B
      - Strophanthus
- B. Purple in Method A
  - 1. Blue in Method C
    - (*a*) Colorless in Method B
      - Clove
      - Myrica
      - Wild Cherry Bark
      - White Oak
    - (*b*) Purple in Method B
      - Ipecac
    - (*c*) Blue in Method B
      - Cardamom
      - White Pine
      - Sassafras
      - Sumbul
    - (*d*) Blue-green in Method B
      - Ipomoea
  - 2. Not blue in Method C
    - (*a*) Colorless in Method B
      - (1) Colorless in Method C
        - Kamala
      - (2) Green in Method C
        - Viburnum prunifolium
    - (*b*) Purple in Method B
      - (1) Blue-green in Method C
        - Leptandra
    - (*c*) Blue in Method B
      - (1) Green in Method C
        - Aspidosperma
    - (*d*) Green in Method B
      - (1) Colorless in Method C
        - Pulsatilla
      - (2) Violet in Method C
        - Scutellaria
      - (3) Green in Method C
        - Cimicifuga
- C. Green in Method A
  - 1. Blue in Method C
    - (*a*) Green in Method B
      - Guaiac
    - (*b*) Yellow in Method B
      - Caulophyllum
  - 2. Blue-green in Method C
    - (*a*) Blue in Method B
      - Coto
    - (*b*) Yellow-green in Method B
      - Asarum
  - 3. Green in Method C
    - (*a*) Colorless in Method B
      - Haematoxylon
    - (*b*) Blue-green in Method B
      - Lupulinum
- D. Spots and another color in Method A
  - 1. Purple color in Method A
    - (*a*) Colorless in Method B
      - (1) Blue in Method C
        - White Oak
  - 2. Red in Method A
    - (*a*) Colorless in Method B
      - (1) Colorless in Method C
        - Pilocarpus
      - (2) Purple in Method C
        - Castanea
    - (*b*) Blue spots in Method B
      - (1) Violet in Method C
        - Spearmint
- E. Spots only in Method A
  - 1. Colorless in Method B
    - (*a*) Colorless in Method C
      - Physostigma
    - (*b*) Blue-green in Method C
      - Granatum
    - (*c*) Green in Method C
      - Areca
      - Frangula
  - 2. Blue in Method B

Key for Identification of Powdered Vegetable Drugs on Basis of Their Fluorescence in Ultraviolet Radiations

(a) Blue in Method C
Red cinchona
3. Green in Method B
(a) Purple in Method C
Scoparius
4. Yellow spots in Method B
(a) Blue in Method C
Ceylon cinnamon

F. Yellow in Method A
1. Yellow in Method B
(a) Blue in Method C
Berberis
Calumba
Hydrastis
Xanthoxylum
(b) Orange in Method C
Curcuma

G. Orange in Method A
1. Blue in Method C
(a) Yellow in Method B
Red Saunders
(b) Orange in Method B
Sanguinaria
2. Green in Method C
(a) Colorless in Method B
Rhubarb

H. Red in Method A
1. Colorless in Method C
(a) Colorless in Method B
Cataria
Lobelia
Pilocarpus
Pimenta
(b) Purple in Method B
Cubeb
2. Violet in Method C
(a) Colorless in Method B
Belladonna leaf
Hamamelis leaf
Peppermint
Senna
Stramonium
Uva Ursi
(b) Green in Method B
Digitalis
Matico
(c) Brown in Method B
Hyoscyamus
(d) Blue spots in Method B
Spearmint
3. Purple in Method C
(a) Colorless in Method B
Salvia
Castanea
4. Blue in Method C
(a) Colorless in Method B
Sabina
(b) Blue in Method B
Coccus
5. Green in Method C
(a) Colorless in Method B
Buchu
Chimaphila
Trifolium

II. Colorless in Method A
A. Colored in Method C
1. Blue in Method C
(a) Colorless in Method B
Anise
Belladonna root
Colchicum root
Colchicum seed
Conium
Convallaria
Coriander
Phytolacca
Stillingia
Viburnum opulus
(b) Blue in Method B
Hydrangea
Krameria
Piper
(c) Blue-green in Method B
Willow
(d) Green in Method B
Jalap
Licorice
(e) Purple in Method B
Grindelia
Nutmeg
2. Blue-green in Method C
(a) Colorless in Method B
Aconite
Apocynum
Cantharis
Capsicum
Cornus
Eucalyptus
Gossypium
Juglans
Mace
Matricaria
(b) Green in Metho
Calendula
3. Green in Method C
(a) Colorless in Method B
Bitter Orange Peel
Blue flag
Canella
Caraway
Chenopodium
Cocculus
Ergot
Eriodictyon
Saigon Cinnamon
Santonica
Sarsaparilla
Zingiber
(b) Blue in Method B
Aspidium
Sabadilla
(c) Blue-green in Method B
Gambir
Serpentaria
Taraxacum
(d) Green in Method B
Valeriana
(e) Yellow-green in Method B
Sambucus
4. Yellow in Method C
(a) Colorless in Method B
Cascara Sagrada
(b) Green in Method B
Foenugreek
(c) Yellow in Method B
Althaea

(*Continued on Page 328*)

Key for Identification of Powdered Vegetable Drugs on Basis of Their Fluorescence in Ultraviolet Radiations (*Continued from Page 327*)

B. Colorless in Method C

1. Colorless in Method B

Arnica
Asclepias
Bryonia
Calamus
Colocynth
Elm
Euonymus
Eupatorium
Illicium
Sandalwood

2. Blue-green in Method B

Agaricus
Foeniculum

3. Green in Method B

Inula
Pyrethrum flowers
Thyme

4. Yellow-green in Method B

Lappa

5. Yellow in Method B

Gentian
Orris
Quassia

DISCUSSION

The study of fluorescence of powdered drugs as a means of effecting their identification appears to possess distinct possibilities of practical application, especially in the case of similar drugs that may be more or less difficult to distinguish. For example, Ceylon cinnamon can be quickly and reliably distinguished from other common varieties such as China and Saigon by virtue of the fluorescent spots that appear when it is mounted as in either Method A or B whereas the latter two varieties are nonfluorescent in Methods A and B. All three varieties fluoresce in method C but Ceylon cinnamon fluoresces blue whereas the China and Saigon cinnamons both appear green. The latter two may be separated, however, by the difference in intensity of fluorescence. In all cases that require use of estimates of intensity of fluorescence for identification comparison of the unknown should be made with a reference standard slide prepared with a sample of known identity.

Satisfactory segregation of the drugs into comparatively small groups, and in many instances final identification, can be made readily by means of the accompanying key. For the most accurate work, however, one should prepare a color code table in which a definite designation of the color of fluorescence of identified drugs in each method of mounting is recorded. Then if a guide such as Ridgeway (15) or Séguy (16) is used to evaluate the colors it should be necessary to resort to estimates of intensity only occasionally. We do not yet have great confidence in identifications that are based entirely on estimates of intensity, since in some of these cases the intensity of fluorescence is none too bright even under the most favorable circumstances. The fluorescence method may prove valuable, nonetheless, for identification even of those drugs which show duplication of color, since there are seldom more than four or five drugs in each color group even when only relatively crude separations are made. Thus, an unknown may be placed rapidly as one of a small group of drugs that can then be examined minutely by the conventional methods in a comparatively short time. This avoids the necessity of considering a large number of drugs microscopically. Frequently the drugs in such a group are so dissimilar in histological characteristics that only a cursory examination is required to identify them microscopically after the primary separation on the basis of fluorescence has been made. When mounted in the same way a large number of drugs may emit the same wave lengths under excitation by near ultraviolet radiations, but generally comparison of their fluorescence in the three methods of mounting (A, B, and C) permits differentiation. Further refinements of technique and use of different solvents should be expected to make possible indubitable identification of each of the several drugs that fall into a single group in the present key. For example, use of a diffraction grating might be expected to reveal subtle differences in the colors emitted by different drugs that appear to the unaided eye to emit the same color. Introduction of a fluorimeter to accurately measure quantitative differences in intensity of fluorescence might prove helpful.

There appear to be two principal difficulties that may be encountered in the fluorescence procedure for identifying crude drugs. The first is that which might occur in discriminating between colors, between blue-green and blue, for example. Obviously, if an error were made in the primary separation, the investigator would proceed through part of the key inapplicable to the particular drug under examination, and a satisfactory identification could not be achieved. However, this difficulty is no greater in this method than is that encountered in any dichotomous or "multiple-choice" key. An individual with a normal sense of color discrimination should have no trouble on this score.

The most serious defect in the present system appears to be its inapplicability to the ten drugs that do not fluoresce in any of the methods tested and to many of the leaves which fluoresce red in Method A, as might be expected because of their chlorophyll content, and which show very similar color changes in Methods B and C. Evidence from experiments now continuing in this laboratory indicates that this problem is amenable to satisfactory resolution by the use of appropriate reagents to intensify differentially or quench fluorescence or to induce qualitative differences in the emission. For example, in one series of experiments, Belladonna, Hyoscyamus, and Stramonium were chosen for study. Experiments were performed with the powdered drug and with aqueous and alcoholic extracts. Two satisfactory tests were finally obtained employing alcoholic extracts prepared by using powdered drug and 95% ethyl alcohol in the proportions of 0.5 Gm. to 10 ml. The suspension was shaken for ten or fifteen minutes and then set aside for twenty-four hours in a tightly stoppered container at room temperature. Then it

was filtered and made up to the original volume with alcohol and tested as outlined below.

The reagents used in the tests that followed were:

I. $AgNO_3$.....Saturated aqueous solution
II. NaOH.....0.1 *N* aqueous solution
III. $HgCl_2$.....5% aqueous solution

Test No. 1.

1. Add 1 drop of Reagent I to 2 ml. of extract and note color of fluorescence under U.V., then
2. Add 3 drops of Reagent II to extract containing I and note color of fluorescence under U.V.

Test No. 2

1. Add 2 drops of Reagent II to 2 ml. of extract and note color of fluorescence under U.V., then
2. Add 1 drop of Reagent III to extract containing II and note color of fluorescence under U.V., then
3. Add 1 more drop of III and note color of fluorescence under U.V., and then
4. Add 2 more drops of III and again note fluorescence as above.

The colors emitted at the various stages of treatment are shown in Table I.

the wave lengths emitted when the extracts are exposed to wavelengths in the near ultraviolet. Although further work is required, the results recorded to date indicate that a reliable system of classification based on fluorescence can be developed for the precise identification of the relatively few drugs, the resolution of which is questionable when classified according to the tentative key given herewith.

Aqueous extracts were prepared in the same manner as the alcoholic extracts, except that the suspensions were heated for ten minutes on a water bath and then were permitted to stand overnight. Addition of a solution of potassium ferrocyanide resulted in a distinctive change in the color of fluorescence only in the case of Belladonna. Other reagents tested were ineffective.

The results obtained in these tests show the importance of studying the role that the solvent and method of preparing the sample may play in determining or influencing the color and intensity of fluorescence. The work of Zechner and Gstirner (5, 6) emphasized this. Leupin (8) and Leupin and Steiner (12) also commented on this subject. Good-

TABLE I.—COLOR CHANGES IN FLUORESCENCE OF ALCOHOLIC EXTRACTS OF THREE POWDERED DRUGS AT DIFFERENT STAGES OF TESTS NOS. 1 AND 2 (SEE TEXT)

Step	Reagent	Belladonna	Hyoscyamus	Stramonium
		Test No. 1		
1	1 drop No. I	Purple	Red	Red (slightly darker than Hyoscyamus)
2	3 drops No. II	Blue	Green	Brown
		Test No. 2		
1	2 drops No. II	Blue	Red	Red
2	1 drop No. III	Purple	Brown	Red
3	1 drop No. III	Bright blue-green	Gray	Red
4	2 drops No. III	Blue	Gray-green	Red-violet

The order of adding the reagents and their concentrations are important, as are the time intervals between additions of reagents and observation of fluorescence. In general, the colors darken with time and eventually become indistinguishable. An interval of approximately one minute between steps has been found convenient and satisfactory. For best results it is desirable to run known identified samples along with the unknowns under test. Preliminary trials have shown that stock alcoholic extracts may be kept for several weeks as reference standards without deterioration of fluorescence characteristics. Stability tests to determine how long such solutions may be kept without deteriorating are in progress. If it is certain that the drug in question is one of these three, i.e., Belladonna, Hyoscyamus, or Stramonium, an individual familiar with the tests can confidently make an identification without use of the reference standards. Both Tests 1 and 2 should be used, however, to provide a double check.

The tests listed above were the best ones found, but several other reagents also resulted in changing the fluorescence of the alcoholic extracts. These reagents included KCNS, $KMnO_4$, $(NH_4)_2MoO_4$, $AuCl_3 \cdot 2H_2O$, and tannic acid. These tests are not satisfactory and are included here only to indicate the wide variety of compounds that may be used to induce changes in

win and Kavanagh (17) studied the fluorescence of acetone and of *n*-butanol extracts of roots of 135 species of plants distributed in 69 families and reported that nearly all of them emitted a bluish or white color. None was reported as emitting predominantly red or orange and only one was reported as having a yellow tint, whereas in our investigation 85% of the drugs that appeared yellow or orange in Method A were underground structures, i.e., roots, rhizomes, or corms, and a number were found that were red or green. Linsbauer (18) using different techniques reported yellow fluorescence in roots of legumes. The importance of the solvent can be further illustrated by the following example. Cascara Sagrada appears yellow in Method C. Substitution of ethanol, ethylene glycol, propylene glycol, or glycerin for methanol causes no readily apparent change in quality or intensity of fluorescence. If water, ether, benzene, or isopropyl alcohol are used as solvents, however, the drug fluoresces green when exposed to the ultraviolet radiation; if acetone is used it appears blue, and blue-green if chloroform is used. Other factors that are known to influence fluorescence characteristics and which should be studied in connection with investigations aimed toward developing a system of identification are *p*H and temperature (19) and perhaps age of the sample (20). It has been reported (20) that the fluorescence

of various dried food products changes with time of storage.

Although a correlation was noted between color of fluorescence and part of the plant used in this work, no correlation was observed between fluorescence and taxonomic position of the plant. Another outcome of this line of investigation might be further elucidation of the nature of some of the more obscure chemical constituents of crude drugs.

SUMMARY

The fluorescence of 151 identified powdered drugs exposed to near ultraviolet radiations, predominantly the 3650 line from a mercury arc, was studied. Drugs were prepared in three different ways for examination, i.e. (*a*) mounted in a solution of nitrocellulose in amylacetate, (*b*) treated for 30 minutes with 1 *N* NaOH in methanol and then mounted as in (*a*), and (*c*) mounted in NaOH as in (*b*) and examined immediately. Observations made on drugs mounted in these three ways led to development of a key for identification of powdered vegetable drugs based on their fluorescent properties. The possible advantages of such a system of identification and the weaknesses of the present key are pointed out.

APPENDIX

It has already been stated (page 328) that experimental results emanating from different techniques indicated that a number of leafy drugs which exhibit similar responses in Methods A, B, and C might be successfully distinguished from each other by means of two simple tests applied to alcoholic extracts of

TABLE II.—SUPPLEMENTAL TESTS WITH ALCOHOLIC EXTRACTS INTENDED TO DISCRIMINATE BETWEEN DIFFERENT VARIETIES OF CERTAIN DRUGS

Drug	Untreated (Alc. Ext.)	Test No. 1 Step No. 1 ($AgNO_3$)	Test No. 1 Step No. 2 (NaOH)	Test No. 2 Step No. 1 (NaOH)	Test No. 2 Step No. 2 ($HgCl_2$)
		(4 drops)	(3 drops)	(6 drops)	
Benzoin, Siam	Blue	Greenish	Dark green	Dark blue	.....
Benzoin, Sumatra	Blue	Greenish	Light green	Light blue	.....
Buchu, Long	Purple	.....	.....	.....	.....
Buchu, Short	Blue	.....	.....	.....	.....
		(5 drops)	(2 drops)	(3 drops)	(2 drops)
Cinchona, Red	Bright blue	Bright blue	Blue	Blue	Purple
Cinchona, Yellow	Blue	Blue	Dark blue-green	Green	Brown-black
		(2 drops)	(1 drop)	(4 drops)	(1 drop)
Cinnamon, Ceylon	Gray-blue	Gray-blue	Gray	Gray	Violet
Cinnamon, China	Gray-blue	Gray-blue	Blue-green	Light gray	Blue
Cinnamon, Saigon	Gray-blue	Gray-blue	Gray	Blue-green	Brown
		(1 drop)	(1 drop)	(3 drops)	
Ipecac, Cartagena	Bright blue	Bright blue	Light blue	Bright green	Light blue
Ipecac, Rio	Bright blue	Bright blue	Dark blue	Dark green	Dark blue
		(3 drops)	(1 drop)	(3 drops)	
Licorice, Russian	No fluorescence	No fluorescence	Brown	Faint dark green	.....
Licorice, Spanish	No fluorescence	Faint green	Green	Bright green	.....
		(6 drops)	(1 drop)	(2 drops)	(2 drops)
Mustard, Black	Bright blue	Blue	Dark blue	Green	Green
Mustard, Yellow	Bright blue	Bright blue	Light blue	Bright yellow-green	Bright green
				(10 drops)	(6 drops)
Pepper, Black	Dull blue	.....	.....	Dull blue	Light blue-green
Pepper, White	Dull blue	.....	.....	Dark blue-green	Dark green
					(Lacto-phenol—2 drops)
		(4 drops)	(1 drop)	(7 drops)	
Plantago Seed, Blonde	Bright blue	Bright blue	Dark blue	Bright green	Blue
Plantago Seed, French	Bright blue	Bright blue	Dark violet	Bright green	Violet
		(1 drop)	(3 drops)	(2 drops)	(6 drops)
Senna, Alexandrian	Red	Red	Green	Red	Brown
Senna, Indian	Red	Red	Light green	Red	Green
		(1 drop)	(2 drops)	(10 drops)	(8 drops)
Xanthoxylum, Northern	Bright blue	Bright blue	Blue	Bright blue	Bright blue
Xanthoxylum, Southern	Bright violet	Purple	Purple	Dull blue-gray	Dark purple

TABLE III.—COLOR CHANGES IN FLUORESCENCE OF ALCOHOLIC EXTRACTS OF LEAFY DRUGS AT DIFFERENT STAGES OF TESTS NOS. 1 AND 2

Drug	Plain (Alc. Ext.)	Test No. 1 Step No. 1 (1 Drop $AgNO_3$)	Test No. 1 Step No. 2 (3 Drops NaOH)	Test No. 2 Step No. 1 (2 Drops NaOH)	Test No. 2 Step No. 2 (1 Drop $HgCl_2$)	Test No. 2 Step No. 3 (1 More Drop $HgCl_2$)	Test No. 2 Step No. 4 (2 More Drops $HgCl_2$,)
Belladonna	Red	Purple	Blue	Blue	Purple	Bright blue-green	Blue
Buchu, Long	Purple	Dark purple	Dark blue	Purple	Purple	Purple	Gray
Cataria	Bright pink	Bright pink	Dull violet	Bright pink	Bright pink	Bright pink	Light gray
Chimaphila	Red-violet	Red	Brown-red	Red-purple	Red-purple	Red-purple	Red-purple
Digitalis	Red-violet	Violet	Dark purple	Violet	Violet	Purple	Purple
Eriodictyon	Blue	Dark blue	Black	Blue	Blue	Blue	Blue
Eucalyptus	Light brown	Brown	Dark brown	Brown	Brown	Brown	Brown
Hamamelis Leaf	Red	Dark red	Dark red	Red	Red	Red	Red
Hyoscyamus	Red	Red	Green	Red	Brown	Gray	Gray-green
Lobelia	Bright violet	Bright purple	Dull blue	Bright violet	Blue	Blue	Blue
Matico	Red	Dark red	Dark brown	Red	Red-brown	Brown	Brown
Peppermint	Red	Red	Dark red-purple	Orange-red	Orange-red	Orange-red	Orange-red
Pilocarpus	Tan	Brown	Green	Tan	Tan	Tan-gray	Gray
Sabina	Red-violet	Purple	Blue-green	Purple	Purple	Purple-gray	Gray
Salvia	Red-violet	Red-violet	Purple	Red-violet	Red-violet	Red-violet	Purple
Senna	Light brown	Light brown	Green	Light brown	Green	Green	Green
Spearmint	Red	Red	Brown-black	Red	Red-purple	Purple-brown	Dark gray
Stramonium	Red	Red	Brown	Red	Red	Red	Red-violet
Uva Ursi	Purple-brown	Purple-brown	Red-purple	Purple-brown	Purple-brown	Purple-brown	Purple-brown

drugs. An example using Belladonna, Hyoscyamus, and Stramonium was cited (page 329). The purpose of this appendix is to present further evidence to support that view. These confirmatory tests are offered as a means of clarifying the classification and identification of some of the crude drugs, determination of which was questionable in Methods A, B, and C. The results of the tests are presented in the form of an appendix because it is believed that their inclusion in the body of the text would impair its continuity and coherence.

The techniques that were used are described on page 329. These tests make it possible not only to distinguish many leafy drugs from each other but they possess the added advantage that with suitable modification (indicated in Table II) they may be used to discriminate between different varieties of a number of crude drugs, such as, for example, between the red and yellow varieties of Cinchona, between the French and blonde forms of Plantago Seed, between different varieties of Ipecac, of Senna, of Licorice, and of Mustard, to mention only a few. In some cases the response of the alcoholic extract alone or treated with only one of the reagents is so distinctive that it is unnecessary to resort to the use of the three reagents listed on page 329. In the case of Plantago Seed it was helpful to substitute lactophenol for $HgCl_2$ in Test No. 2. The results of these several tests are shown in Tables II and III.

Since sodium hydroxide is used in these tests attention should be directed to the marked influence that hydrogen-ion concentration exerts on the fluorescence of liquid preparations (cf. Radley, J. A., and Grant, J., "Fluorescence Analysis in Ultraviolet Light," ed. 3, D. Van Nostrand Company, New York, 1939, p. 424.

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Sample Size of Parenteral Solutions for Sterility Testing*

By LILA F. KNUDSEN†

Two questions have been asked in regard to sampling for sterility testing: "What size of representative sample should be taken?" and "How much should sample size vary with lot size?" Both of these questions can be answered by using some mathematical calculations together with a few assumptions. Recent developments in sampling theory have shown that sometimes tremendous variations in the quality of lots passed are due to chance alone involved in selecting the sample. It has been shown that it is possible to predict the variations and the frequency with which they will occur. The results of the mathematical calculations on a particular sample plan can be shown graphically by means of a curve called an "operating characteristic curve." Much has been written on sampling; however, Grant (1) and the Statistical Research Group (2) summarize sampling developments quite well.

ASSUMING that the bacteriological test is accurate and detects contamination with microorganisms in a container when present, the following "operating characteristic curves" show the probability of passing lots having varying incidence of contamination on the basis of testing a sample of a certain number of containers without finding contamination. For instance, if a sample of 10 containers is tested from the lot that is known to be 10 per cent defective, what are the chances of finding no contamination in the sample and therefore passing the lot? Of course, if a sample is tested from a lot having no contamination, the lot will always be accepted.

First, possibly, a definition of the term "operating characteristic curve" is in order. It is a curve plotted from mathematical calculations[1] showing the percentage of defects in, or quality of, the *lot* (not the sample) on the horizontal scale and the probability of accepting the lot on the vertical scale. All of the operating characteristic curves shown here are based on the testing of a specified number of containers (or units) from the lot and rejecting the lot if *one* container in the sample tested is found contaminated. In other words, the lot is accepted only if all containers are found to be free of contamination. From the operating characteristic (or *OC*) curve it is possible to see what chances are being taken of passing a lot containing a given percentage of defectives in the *lot*.

The curve on Fig. 1 labeled $N = 100$, $n = 10$ is the *OC* curve for the sampling specification: *From a lot of 100 containers take a sample of 10; if no container is found to be contaminated, pass the lot.* (The lot size is indicated by N, the sample size by n on all figures.) This curve shows that such a sampling plan would result in passing 1 lot out of every 3 lots having 10 per cent of the containers in the *lot* (not the sample) contaminated. The probability is thus 34 out of 100 that a 10 per cent defective *lot* will be accepted if decision on whether or not to accept the lot is based on testing 10 containers and finding no contamination. The chances of accepting a lot in which 20 per cent are defective (contaminated) are 1 in 10, and of accepting a 30 per cent defective lot are 2 in 100. One time in 200 even a 40 per cent defective lot will be accepted.

Figure 2 shows the *OC* curves for sample size of approximately 15 from lots of differing sizes. The sample sizes corresponding to given lot sizes are:

$N = 50$	$n = 13$
$N = 100$	$n = 14$
$N = 300$	$n = 15$
$N = 500$	$n = 15$
$N = 10{,}000$	$n = 15$

By increasing the sample size from 10 to about 15 it can be seen that the probability of accepting a *lot* containing 10 per cent defectives is decreased from 34 out of 100 to 19 out of 100 and the probability of accepting a *lot* containing 20 per cent defectives is 3 out of 100.

Figure 3 shows the *OC* curves for samples of about 20. Here:

$N = 50$	$n = 17$
$N = 100$	$n = 18$
$N = 300$	$n = 20$
$N = 500$	$n = 20$
$N = 10{,}000$	$n = 20$

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[1] The probability of acceptance is calculated for several distinct points on the curve by means of the formula

$$\text{Prob. of accept.} = \frac{(N-d)!(N-n)!}{(N-n-d)!N!}$$

where N = lot size, n = sample size, d = number of defects in the lot, and ! = factorial [e.g., $4! = 4(3)(2)(1) = 24$].
For instance for $N = 100$, $n = 10$, and $d = 5$

$$\text{Prod. of accept.} = \frac{95!90!}{85!100!} = 0.584$$

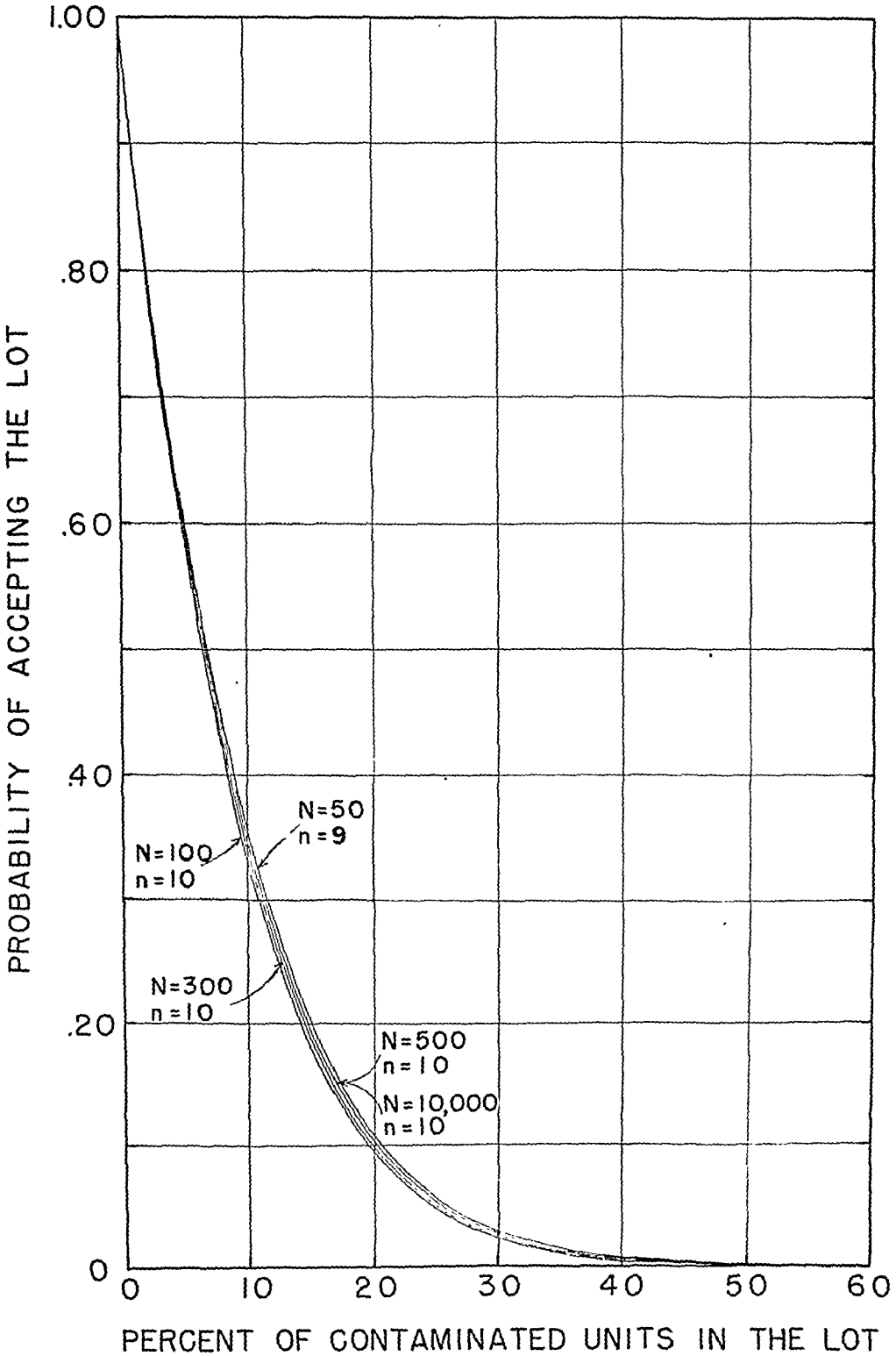

Fig. 1.—Operating characteristic curves for several lot sizes (N) and sample size of about 10 units. The lot is passed if there are no contaminated units in the sample tested.

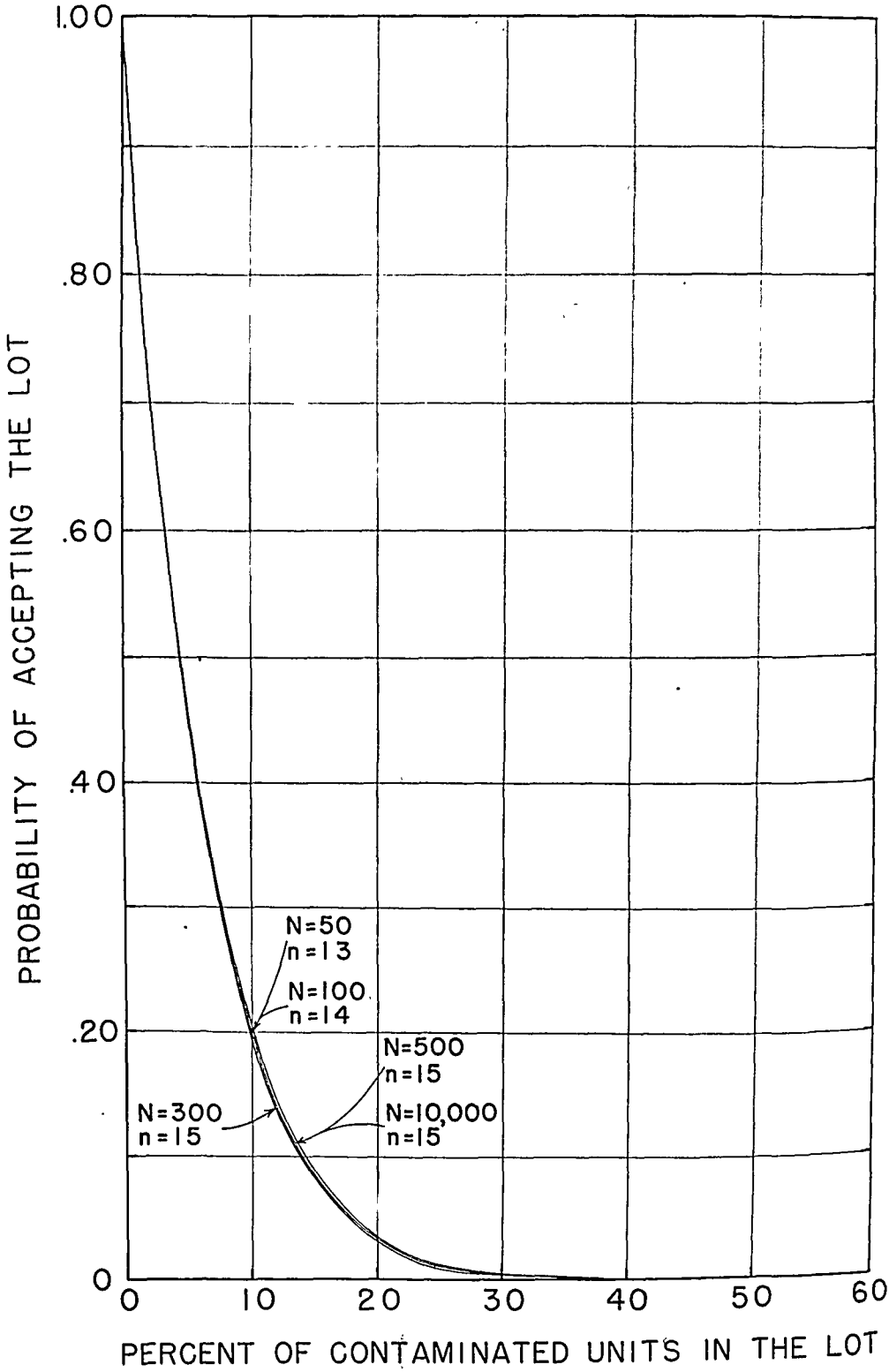

Fig. 2.—Operating characteristic curves for several lot sizes (N) and sample size of about 15 units. The lot is passed if there are no contaminated units in the sample tested.

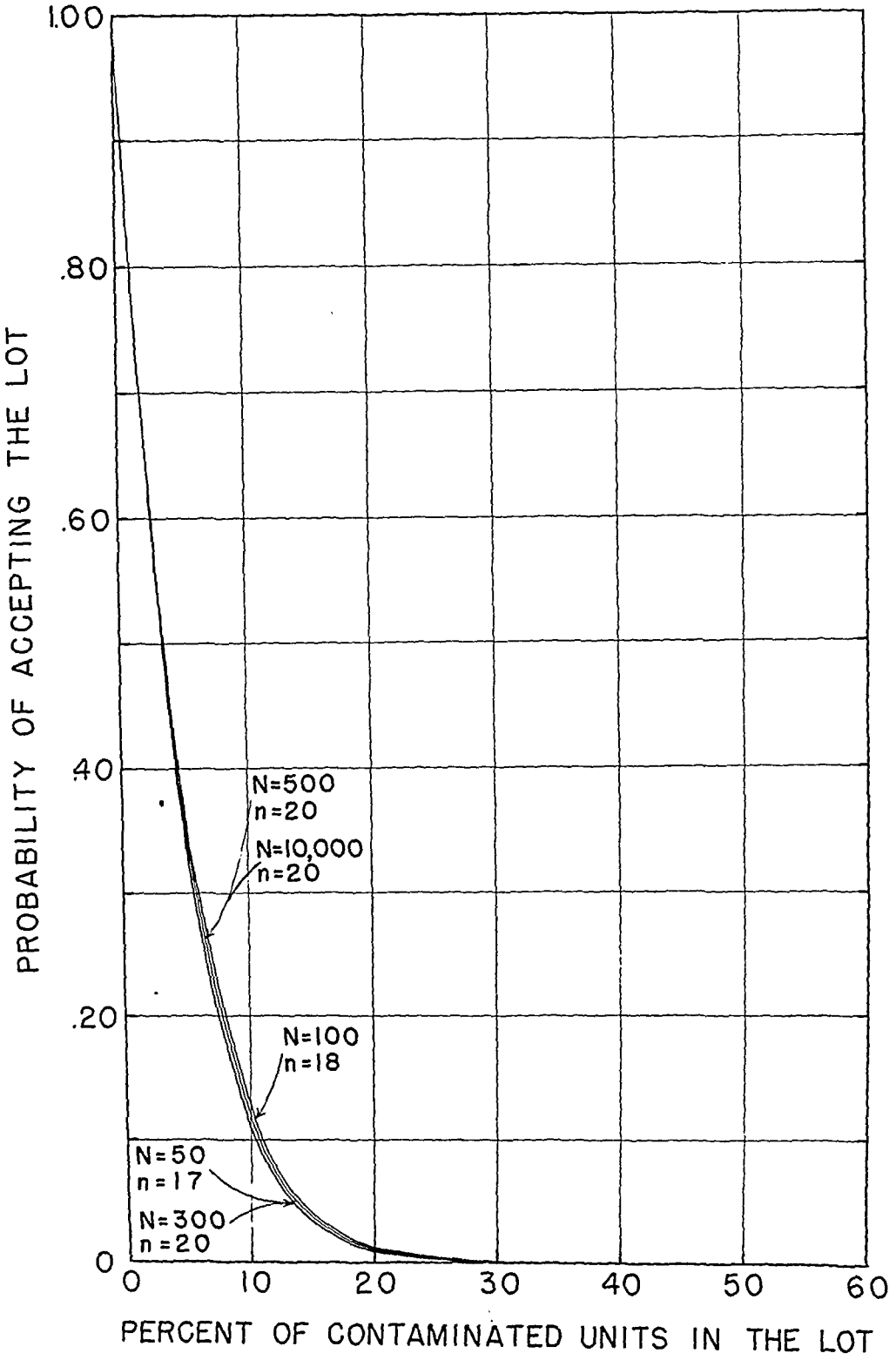

Fig. 3.—Operating characteristic curves for several lot sizes (N) and sample size of about 20 units. The lot is passed if there are no contaminated units in the sample tested.

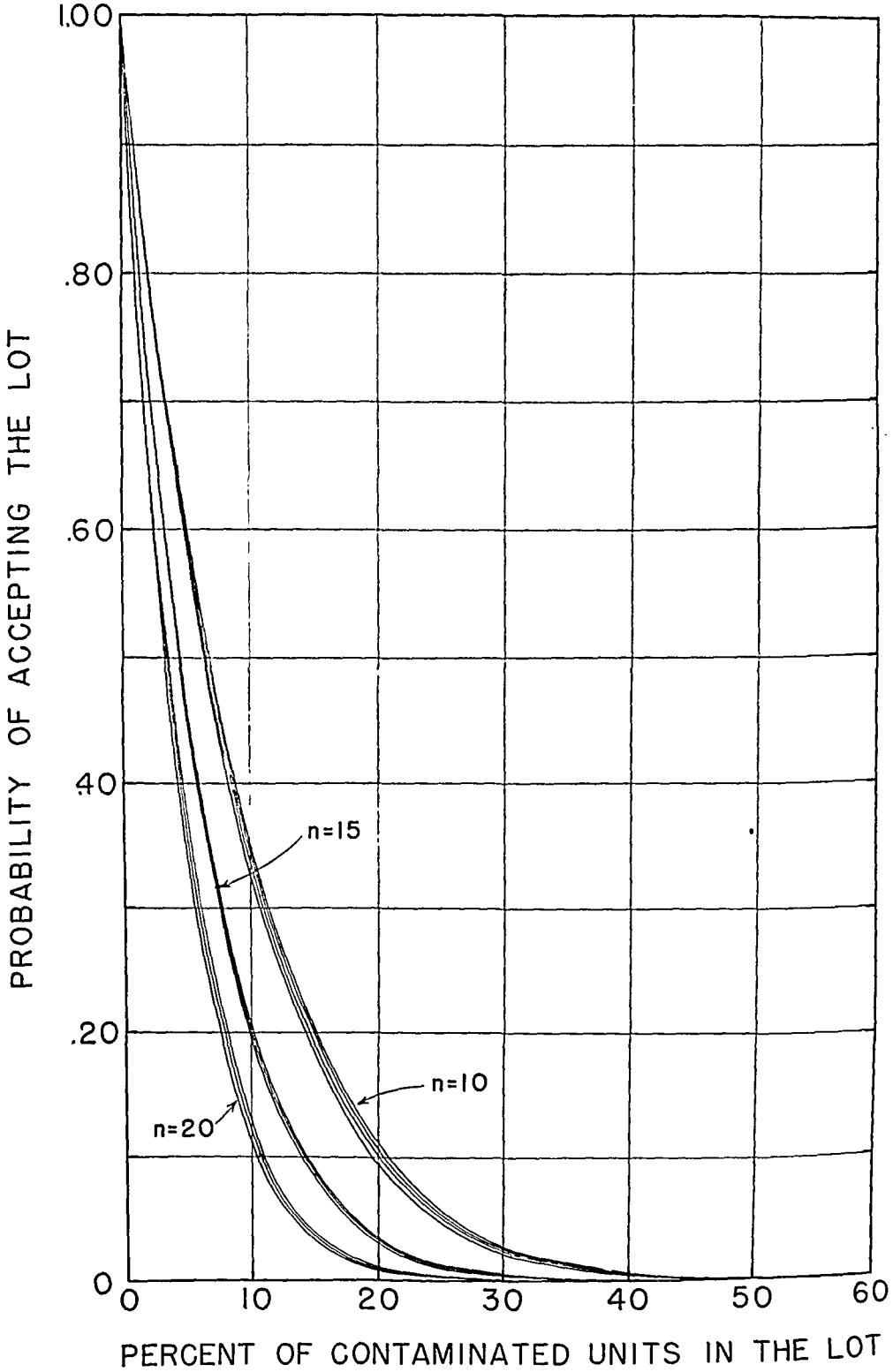

Fig. 4.—Operating characteristic curves for several lot sizes (N) and respective sample sizes (n). The lot is passed if there are no contaminated units in the sample tested. (Fig. 4 is a composite of Figs. 1, 2, and 3.)

The probability of accepting a *lot* containing 10 per cent defectives is still very large—namely, 1 in 9, and there is still one chance in 100 of passing a lot containing as much as 20 per cent defectives.

Figure 4 combines on one chart the *OC* curves for Figs. 1, 2, and 3. Comparative chances for different sample sizes are shown in Table I.

leniency with small lots than with large lots. For instance, given two lots, one large and one small, each with the same sample size, the respective probabilities of accepting a lot with, perhaps, 10 per cent defective will be the same. If the sample size of the smaller lot is decreased the probability of accepting a lot with, for example, 10 per cent defective is increased.

TABLE I.—CHANCES OF PASSING DEFECTIVE LOTS AS RELATED TO SAMPLE SIZE

% Defective in Lot (% of Contaminated Units in the Lot)	Chances of Passing Lot After Testing Sample of Size: $n = 10$	$n = 15$	$n = 20$
5	60 out of 100	45 out of 100	35 out of 100
10	34 out of 100	20 out of 100	11 out of 100
15	19 out of 100	8 out of 100	4 out of 100
20	10 out of 100	3 out of 100	1 out of 100
30	2 out of 100	5 out of 1000	1 out of 1000

Mathematical calculations were made to determine the size of sample to be taken from lots of 50, 100, 300, and 500 containers in order that the *OC* curve will most closely approximate that for $N = 10{,}000$ and $n = 10$. From these calculations it was found that the same size of sample ($n = 10$) should be taken for $N = 300$, 500, or 10,000 and that for $N = 50$, $n = 9$. As is obvious from Figs. 1, 2, and 3, the *OC* curves for approximately the same size sample are practically identical over a wide range of lot sizes. This means that the probability of accepting lots having a given percentage of contaminated containers is directly related to sample size rather than lot size.

If sample size increased directly or proportionately with lot size, it would result in greater

Thus decreasing the sample size with decreasing lot size would result in increasing the probability of passing lots having a given percentage defective. It would also result in passing lots having a greater percentage defective.

The inescapable conclusion is reached that since there is no reason to be more lenient with a small lot than with a large lot, the sample size taken must be approximately the same regardless of lot size.

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Analysis of Diisopropylfluorophosphate in Oil Solution*

By C. R. SZALKOWSKI, W. J. MADER, and H. A. FREDIANI

A combination of a total phosphorus determination with a so-called free hydrofluoric acid determination in the analysis of diisopropylfluorophosphate (DFP) in oil solution is described and discussed. The first part of this combination of determinations indicates initial DFP content while the second reveals the degree of hydrolysis.

ONE OF THE TOXIC materials investigated for wartime usage (1, 2) which has shown itself to be useful pharmacologically (3, 4) is diisopropylfluorophosphate (DFP). Because this chemical is strongly susceptible to hydrolysis, aqueous solutions have been found to vary in potency and to lose potency fairly rapidly (5). Since it is quite stable in oil solution, this drug has been introduced as a myotic and a 0.1 per cent concentration in peanut oil is sufficient to counteract a 4 per cent solution of homatropine bromide (3).

PROCEDURE

The problem involved in the adequate analysis of such oil solutions is extremely interesting from a

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chemical point of view. The hydrolysis proceeds according to the following equation:

$$2(C_3H_7O)_2POF + 2H_2O \rightarrow H_2F_2 + 2(C_3H_7O)_2POOH$$

resulting in the formation of an organic phosphate and hydrofluoric acid, neither of which apparently shows significant pharmacological efficacy.

Because of the low precision of animal tests, a chemical method for indicating the actual composition at a given time is highly desirable. Since degradation in oil solution proceeds slowly, precise chemical studies are required in order to evaluate properly the storage stability of such a product. A combination of a total phosphorus determination, to indicate initial DFP content of the preparation, together with a "free hydrofluoric acid" determination, to indicate degree of hydrolysis, has proved of value in our laboratory.

Determination of Initial DFP Content.—The total phosphorus present is converted to inorganic pyrophosphate by ignition in the presence of zinc oxide. Because of the small quantities involved, the colorimetric method for phosphorus originally reported by Eddy and De Eds (6) as modified for use in oils by Goodloe (7) has been found most satisfactory. Following the recommendations in the study by Kitson and Mellon (8), optical density measurements at 640 μ were used for which a photoelectric filter photometer with Corning filter No. 2404 served satisfactorily.

Reagents.—10 *N Sulfuric acid:* 282 ml. of reagent concentrated (sp. gr. 1.84) sulfuric acid diluted to 1 L. *Stannous Chloride:* Prepare a stock solution by dissolving 5 Gm. reagent $SnCl_2 \cdot 2H_2O$ in 25 ml. concentrated hydrochloric acid. For use, dilute exactly 1 ml. of stock solution to 100 ml. with distilled water. The stock solution is stable for at least two weeks; the dilute solution must be prepared fresh daily. *Molybdate Reagent:* Place 64 Gm. of reagent ammonium molybdate in a 1-L. flask; add 400 ml. distilled water and 500 ml. 10 *N* sulfuric acid. Dissolve by shaking and dilute to volume. *Phosphate Standard:* Dissolve 0.4392 Gm. reagent potassium dihydrogen phosphate in 500 ml. water and 5 ml. of 10 *N* sulfuric acid. Add 0.1 ml. of 0.1 *N* potassium permanganate (or sufficient to give a faint pink color) as preservative and dilute to 1 L. The resultant solution contains 0.1 mg. of phosphorus per ml.

EXPERIMENTAL

Accurately weigh 0.5 Gm. of sample into a small porcelain crucible and cover with 1 Gm. reagent zinc oxide. Heat gently over a low flame until the oil is all burned off, then ignite until free of carbon. Allow the crucible to cool and place in a 250-ml. beaker, removing the contents by tapping the crucible against the side of the beaker. Add about 40 ml. water and 10 ml. 10 *N* H_2SO_4. Heat on a hot plate with occasional stirring until solution is complete and then remove the crucible (rinse while removing). Transfer the solution to a 250-ml. flask and dilute to mark. Pipette a 50-ml. aliquot into a 100-ml. volumetric flask; add 0.2 ml. of *p*-nitrophenol indicator; and neutralize with 20% sodium carbonate solution (dropwise). Add 10 *N* H_2SO_4 dropwise until the solution clears and becomes colorless. Swirl after the addition of each drop. After neutralizing, add 10 ml. of the 10 *N* H_2SO_4 followed by 10 ml. of molybdate reagent. Rinse sides of flask until total volume is approximately 90 ml. and then mix thoroughly by swirling. Slowly add (while mixing) 5 ml. of the diluted stannous chloride solution, noting the time this is added. Dilute to 100 ml. and mix thoroughly. Transfer part of the solution to a colorimeter cell and, exactly ten minutes after addition of the stannous chloride, measure the transmission. Read the mg. of phosphorus directly from a calibration curve prepared with the instrument and reagents in use.

Calibration Curve.—Standard phosphate solutions are prepared by adding 0.005, 0.01, 0.02, 0.03, and 0.04 mg. of phosphorus to individual 100-ml. volumetric flasks, diluting to 50 ml. with water, adding 10 ml. 10 *N* H_2SO_4 and 10 ml. molybdate reagent, diluting to 90 ml., swirling, adding 5 ml. dilute stannous chloride, diluting to mark, and mixing. Transmission readings are taken exactly ten minutes after addition of the stannous chloride.

The colorimeter readings may be taken with the instrument set at zero with distilled water. A blank determination should be carried out with all of the reagents used and final colorimeter readings (for samples and calibration curve) should be corrected by subtracting the value of this blank. A new blank correction must be ascertained each time a new reagent is employed in the determination.

Calculation.—Total (initial) mg. DFP = mg. P $\times \frac{184}{31}$. In order to evaluate the efficacy of this procedure for phosphorus, known solutions of phosphate (as potassium dihydrogen phosphate) were added to the porcelain crucible together with peanut oil and put through the entire procedure. Recoveries of these known samples were 98.0, 98.6, 99.7, and 100.7% of theory.

Degree of Hydrolysis.—For the determination of the low fluoride ion concentrations involved, a modification of the ferric acetyl acetone photometric method of Wilcox (9) has been found to be most practical.

Since the test cannot be carried out in nonaqueous media a water extraction is necessary. By using multiple samples the necessary kinetic data may be obtained so that by extrapolating to zero time, the initial degree of hydrolysis may be calculated. A more thorough study of the kinetics of this hydrolysis will be published elsewhere.

Principle.—The hydrofluoric acid is extracted from the oil by shaking with water. The aqueous fluoride solution is then assayed by the colorimetric ferric acetyl acetone method.

Apparatus and Reagents:

Ferric Nitrate; dissolve 1.1 Gm. $Fe(NO_3)_3 \cdot 9H_2O$ in 500 ml. water.

Acetylacetone; dissolve 2.5 Gm. of freshly distilled acetylacetone in 500 ml. water.

Ferric acetylacetone; mix equal volumes of the ferric nitrate and acetylacetone solutions.

Standard fluoride solution; dissolve exactly 220 mg. reagent NaF and dilute to 1 L. 1 ml. = 0.1 mg. F.

Photoelectric cell colorimeter with 480 μ filter.

Colorimetric Ferric Acetyl Acetone Method.—Dissolve 5 ml. of sample in exactly 25 ml. absolute ether in a 125-ml. separatory funnel. Add (pipette)

25 ml. water and shake for thirty minutes. Allow the two layers to separate and draw off the clear, aqueous layer. If the aqueous layer is not perfectly clear (free of oil or ether), pass through a dry filter. To each of three 25-ml. volumetric flasks add exactly 2 ml. of ferric-acetylacetone solution. To one flask add a 10-ml. aliquot of the clear aqueous extract; to the second flask add 10 ml. of the extract, plus 1 ml. of fluoride standard (0.1 mg.); to the third flask, 10 ml. of water alone. Dilute to volume. Mix thoroughly and after ten minutes, read the color in a suitable colorimeter at 480 μ using distilled water for zero density. Calculate the results according to the following equation:

$$F = \frac{(B - S) \times 25 \times 0.1}{(S - X) \times 10 \times \text{weight of sample}}$$

where

F = mg. free fluoride per gram of sample,
B = optical density of reagents,
S = optical density of unknown,
X = optical density of unknown plus 0.1 mg. added fluoride.

The value obtained here is the free fluoride originally present due to hydrolysis of the sample plus free fluoride resulting from the thirty-minute contact with water. A hydrolysis time study has indicated this value to be within 15% of the original fluoride content of the sample. For routine control purposes, this value is sufficiently close to the truth.

To ascertain the efficacy of the fluoride analysis, together with that of the extraction procedure used, a standard solution of hydrofluoric acid in peanut oil was prepared, containing 0.82 mg. H_2F_2 per gram of oil. By the procedure detailed below recoveries of 97.0, 101.0, and 96.6% were effected.

DISCUSSION

A typical example of the data obtained for ascertaining initial hydrolysis is in order. A sample of diisopropylfluorophosphate in peanut oil was shaken with water at room temperature for periods of time varying from five minutes to five hours and the free fluoride determined as above. As may be expected, the free fluoride content increased with time as indicated in the following tabulation:

Time, Min.	Micrograms F
5	7.2
10	7.4
15	7.7
30	8.5
45	10.5
60	12.5
120	14.5
180	17.0
240	18.5
300	19.0

Because of the large excess of water present, it was presumed that this reaction would be a first-order reaction. A plot of log concentration of DFP, left at time t against t, yielded the typical straight line (Fig. 1) to be expected for a first-order reaction. Extrapolation of this line back to zero time indicates an initial hydrolysis of 7% for this particular sample. The reaction rate for this particular reaction is such that for routine testing of production samples a single determination, using a thirty-minute extraction time, suffices to yield a hydrolysis value within 15% of the initial. Analysis of five different lots for experimental evaluation resulted in the data tabulated below:

Sample	Labeled Conc. %, DFP	% DFP Found	% Hydrolyzed
A	0.1	0.100	20
B	0.05	0.047	12
C	0.1	0.093	8
D	0.1	0.101	7
E	0.1	0.100	3

The column labeled "% DFP Found" was obtained by calculation from the phosphorus determination. The "% Hydrolyzed" values are the maximum values obtained by calculation from the free fluoride concentration.

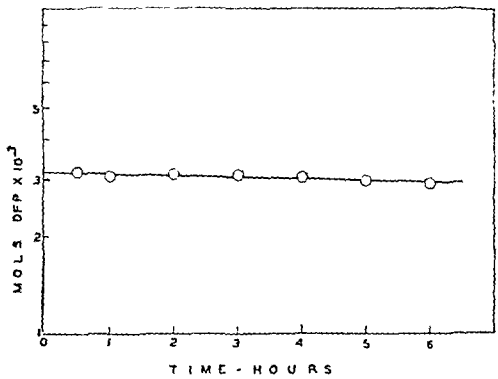

Fig. 1.

SUMMARY

1. A colorimetric phosphorus determination serves to indicate the initial diisopropylfluorophosphate concentration in oil solutions.

2. A colorimetric free fluoride determination indicates the hydrolyzed DFP content of such solutions.

3. The low moisture content of peanut-oil solutions of DFP decreases the rate of hydrolysis so as to result in relatively stable ophthalmic solutions.

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A Brief Historical Survey of Indian Drugs of Vegetable Origin*

By S. PRASAD†,‡

THE HISTORY of the medicinal use of plants in India can be traced to the remotest past. In the *Rigveda*, one of the oldest, if not the oldest, repository of human knowledge, and which is supposed to have come into existence about 4500 B.C., mention has been made of the "Soma" plant, which was given a place similar to the Greek *ambrosia*. In the *Atharva-veda*, which is a later production, the plants and vegetable products, in general, have been fully recognized as helpful agents in the treatment of diseases, though their use takes the form, in many instances, of charms and incantations. Thus the plant "Apamarg" (*Achyranthes aspera*), still considered in the Hindu system of medicine as a diuretic and laxative, is invoked as the "mistress of remedies" and "sole ruler over all plants" (19). It is in the *Ayurveda*, however, the foundation stone of the ancient medical science in India and which is supposed to have been compiled between 2500 and 600 B.C., that definite properties of drugs and their uses have been described in detail.

SCIENCE OF MEDICINE AND PHARMACOGNOSY IN ANCIENT INDIA

The two great works of this age (2500 B.C. to 600 B.C.) are the Charak and the Susrut. While in the Susrut (800 B.C. to 700 B.C.), surgery has been dealt with in great detail, the Charak (1000 B.C. to 800 B.C.) deals more with medicine. The simple medicines alone are arranged by this author under forty-five heads (Chopra, 1933). The methods of administration of drugs are fully described and bear a striking resemblance to those in use at the present time. About 2000 vegetable remedies have been included in the Charak, but only a few mineral drugs and still fewer animal remedies are cited (17). The soil, the season, and the gathering time of individual drugs of the vegetable kingdom are mentioned.

Ayurveda was a subject of study by all learned men, sages, and kings, for the benefit of the human race. The kings in particular encouraged the cultivation of medicinal plants as may b testified from the fact that Ashoka, the benevc lent emperor (274–236 B.C.), established through out his empire, hospitals for men and animals an botanical gardens for the culture of medicina plants as sources of medicine for his people (16)

This was also the period of original researcl which resulted in remarkable progress in every respect. A number of books on *Ayurveda* anc Hindu botany were written. Mention may be made of some of the important works by Vagbhatta, Madhavakar, Chakradatta, Srangdhar, Kanada, Sankarsen, and Bangasen (500 B.C. to 100 B.C.) who elaborated the vegetable *materia medica* of the Hindus and included many more new vegetable drugs. Bhoja-prabandha, a later work, contains a reference to inhalation of medicaments before operation and an anesthetic called "Sammohini" is supposed to have been used in the time of Buddha (4). The Hindu books on Botany, like "Kalpstanum" or "Vrikshayurveda," described detailed characteristics of vegetable drugs, their geographical distribution and habitat, soil suitable for their growth, the season for collecting them, the duration of their efficacy, and the methods of their storage. Elaborate classification of drugs and medicinal plants and detailed instructions on every conceivable point, such as the gathering time for different types of drugs, parts to be collected, manipulations and preparations, and the weights and measures to be used for dispensing, have been given.

HINDU MEDICINE AND ITS INFLUENCE ON FOREIGN COUNTRIES

From the time of Charak and Susrut up to about 1200 A.D., Hindu medicine made very good progress and attained its highest development. It made its way far beyond the shores of India—into Egypt, Greece, and Rome.[1] Dioscorides mentions in his work many Indian plants, particularly the aromatic group of drugs for which India has always been famous. Indeed Indian

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[1] The following quotation from "Culture and Commerce of Cotton in India," by J. Forbes Royle, p. 587 (Smith Elder & Co., 65, Cornhill, London, 1851) offers another aspect of the same fact: "If an indigofera had not been indigenous, indigo would never have derived its name from India, nor have afforded us the proof, in the stripe of mummy-cloth, of the early commercial intercourse between its native country and Egypt. Neither would sugar have been arranged by the Greeks with honeys, . . ."

perfumes, unguents, and similar articles of luxury were then in great demand in Rome and other countries. For instance, Ktesias of Knidos, who was physician to Artaxerxes Mnemon about 400 B.C., refers to cinnamon oil exported from India (26). The anonymous author of *Periplus* states that the export from Sind included *Costus* (*Saussurea lappa*). Pliny also refers to a heavy drain of Roman gold to India in buying costly drugs and aromatic spices (Pliny, "Historia Naturalis," Vol. XXIV, p. 1).

Toward the close of this period, Charak's fame traveled into Arabia. In the eighth or ninth century A.D., Charak's work was translated and was well known in Arabia. Rhazes (865 to 925 A.D.) calls him Scarak, Avicenna (980 to 1035 A.D.) quotes him as Scirak, and Serapion mentions Charak by the name as Zarch. The fact that several standard Hindu works on medicine and *materia medica* were translated into Arabic (Dietz, "Analecta medica"; Wustenfeld, "Geschichte der Arabischem Aerzte" (1840); Fluegel and others), and that several Indian drugs like pepper, lac, nard, licorice, asafetida, *Ocimum sanctum*, bdellium, cinnamon, myrrh, red sandal, Calamus (*Acorus calamus*), and the chebulic myrobalans have been included in the *materia medica* of Useibiah and others, indicates how much Arabic medicine was influenced by the Hindu medicine (Ray, 1902).

POSITION OF HINDU MEDICINE IN MEDIEVAL INDIA

The vegetable drugs employed by the Hindu physician and contained in the various books extant at the period mentioned above ran into several thousands, but during the invasion of India by the Greeks, Scythians, Huns, and the Mohammedans successively, a good deal of the existing Ayurvedic literature was mutilated or lost. Already the Buddhistic doctrine of *Ahimsa* (Non-killing), which led people to consider touching and dissection of dead bodies as sinful, had resulted in a decline of surgery. Medicine, however, had maintained its progress during the Buddhistic period and a large number of vegetable drugs were added to the already extensive *materia medica*. By the eleventh century or so, decadence set in generally, in knowledge, learning, and practice of both medicine and surgery.

When the Mohammedan rule was established in India from the thirteenth century on, the Arabian or the medieval Greco-Arab system, also known as the "Unani-Tibb," became the state system of medication. This system was also fairly advanced for that period, so that the old Hindu system of treatment was pushed to the background. The Arabic system brought with it a rich store of its own *materia medica*, unknown to India. During the long period of Mohammedan rule (up to the seventeenth century), this system came in close contact with the Ayurvedic or the Hindu system of medicine, resulting in a great deal of intermingling.

DECLINE AND EVOLUTION OF THE INDIGENOUS DRUGS OF INDIA

Both the Ayurvedic and the Unani-Tibb systems gradually declined with the fall of the Moghul Empire, coinciding with the advent of the Europeans in India—first the Portuguese, then the French and last the English. When the British had established their rule, the Western system of treatment was introduced in India. This further threw the indigenous systems (Ayurvedic and Unani-Tibb) into the background; nonetheless, both the ancient systems have remained the main source of medical relief to the bulk of the Indian population up to the present.

The modern Western system brought with it its own *materia medica*. Many of the indigenous drugs, however, after some pharmacological, chemical, and clinical investigations, were included in the British Pharmacopœia, and some of these like Kurchi, Ispaghula, Chirata, and Kaladana were accepted by the B. P. 1914. In the "Indian Pharmacopœial List" which has been published under the authority of the Government of India, all these drugs have been given recognition and many more like Rauwolfia, Punarnava, Vasak, etc., have been included. This "List" is considered of the same authority as the supplements to the B. P. published in Canada, Australia, and other dominions.

The earliest attempts at reviving the Indian drugs were confined mainly to the collection of available information about various medicinal plants. Sir William Jones' "Botanical Observations on Select Indian Plants" was one of the earliest contributions in this field (13). This was followed by John Fleming's "Catalogue of Medicinal Plants" (12), Ainslie's "Materia Medica of the Hindustan" (1), Roxburgh's "Flora Indica" (20), and later the works of Wallich (29), Royle (21, 23), and others (Strachey, 1852; Boissier, 1867; Stewart, 1874; Kurz, 1877; and Hooker 1875–97) (28, 2, 27, 15, 10). These did much toward resolving some degree of scientific arrangement from the chaos which existed in the vast mass of India's botanical material.

Of the earlier work on *materia medica* and the pharmacological aspect of Indian drug plants, mention may be made of O'Shaughnessy's "Bengal Pharmacopœia" (18), Irvine's "Materia

A Brief Historical Survey of Indian Drugs of Vegetable Origin*

By S. PRASAD†,‡

THE HISTORY of the medicinal use of plants in India can be traced to the remotest past. In the *Rigveda*, one of the oldest, if not the oldest, repository of human knowledge, and which is supposed to have come into existence about 4500 B.C., mention has been made of the "Soma" plant, which was given a place similar to the Greek *ambrosia*. In the *Atharva-veda*, which is a later production, the plants and vegetable products, in general, have been fully recognized as helpful agents in the treatment of diseases, though their use takes the form, in many instances, of charms and incantations. Thus the plant "Apamarg" (*Achyranthes aspera*), still considered in the Hindu system of medicine as a diuretic and laxative, is invoked as the "mistress of remedies" and "sole ruler over all plants" (19). It is in the *Ayurveda*, however, the foundation stone of the ancient medical science in India and which is supposed to have been compiled between 2500 and 600 B.C., that definite properties of drugs and their uses have been described in detail.

SCIENCE OF MEDICINE AND PHARMACOGNOSY IN ANCIENT INDIA

The two great works of this age (2500 B.C. to 600 B.C.) are the Charak and the Susrut While in the Susrut (800 B.C. to 700 B.C.), surgery has been dealt with in great detail, the Charak (1000 B.C. to 800 B.C.) deals more with medicine. The simple medicines alone are arranged by this author under forty-five heads (Chopra, 1933). The methods of administration of drugs are fully described and bear a striking resemblance to those in use at the present time. About 2000 vegetable remedies have been included in the Charak, but only a few mineral drugs and still fewer animal remedies are cited (17). The soil, the season, and the gathering time of individual drugs of the vegetable kingdom are mentioned.

Ayurveda was a subject of study by all learned men, sages, and kings, for the benefit of the human race. The kings in particular encouraged the cultivation of medicinal plants as may b testified from the fact that Ashoka, the benevc lent emperor (274–236 B.C.), established through out his empire, hospitals for men and animals an botanical gardens for the culture of medicina plants as sources of medicine for his people (16)

This was also the period of original researcl which resulted in remarkable progress in ever respect. A number of books on *Ayurveda* anc Hindu botany were written. Mention may be made of some of the important works by Vagbhatta, Madhavakar, Chakradatta, Srangdhar, Kanada, Sankarsen, and Bangasen (500 B.C. tc 100 B.C.) who elaborated the vegetable *materia medica* of the Hindus and included many more new vegetable drugs. Bhoja-prabandha, a later work, contains a reference to inhalation of medicaments before operation and an anesthetic called "Sammohini" is supposed to have been used in the time of Buddha (4). The Hindu books on Botany, like "Kalpstanum" or "Vrikshayurveda," described detailed characteristics of vegetable drugs, their geographical distribution and habitat, soil suitable for their growth, the season for collecting them, the duration of their efficacy, and the methods of their storage. Elaborate classification of drugs and medicinal plants and detailed instructions on every conceivable point, such as the gathering time for different types of drugs, parts to be collected, manipulations and preparations, and the weights and measures to be used for dispensing, have been given.

HINDU MEDICINE AND ITS INFLUENCE ON FOREIGN COUNTRIES

From the time of Charak and Susrut up to about 1200 A.D., Hindu medicine made very good progress and attained its highest development. It made its way far beyond the shores of India—into Egypt, Greece, and Rome.[1] Dioscorides mentions in his work many Indian plants, particularly the aromatic group of drugs for which India has always been famous. Indeed Indian

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[1] The following quotation from "Culture and Commerce of Cotton in India," by J. Forbes Royle, p. 587 (Smith Elder & Co., 65, Cornhill, London, 1851) offers another aspect of the same fact: "If an indigofera had not been indigenous, indigo would never have derived its name from India, nor have afforded us the proof, in the stripe of mummy-cloth, of the early commercial intercourse between its native country and Egypt. Neither would sugar have been arranged by the Greeks with honeys, . . ."

perfumes, unguents, and similar articles of luxury were then in great demand in Rome and other countries. For instance, Ktesias of Knidos, who was physician to Artaxerxes Mnemon about 400 B.C., refers to cinnamon oil exported from India (26). The anonymous author of *Periplus* states that the export from Sind included *Costus* (*Saussurea lappa*). Pliny also refers to a heavy drain of Roman gold to India in buying costly drugs and aromatic spices (Pliny, "Historia Naturalis," Vol. XXIV, p. 1).

Toward the close of this period, Charak's fame traveled into Arabia. In the eighth or ninth century A.D., Charak's work was translated and was well known in Arabia. Rhazes (865 to 925 A.D.) calls him Scarak, Avicenna (980 to 1035 A.D.) quotes him as Scirak, and Serapion mentions Charak by the name as Zarch. The fact that several standard Hindu works on medicine and *materia medica* were translated into Arabic (Dietz, "Analecta medica"; Wustenfeld, "Geschichte der Arabischem Aerzte" (1840); Fluegel and others), and that several Indian drugs like pepper, lac, nard, licorice, asafetida, *Ocimum sanctum*, bdellium, cinnamon, myrrh, red sandal, Calamus (*Acorus calamus*), and the chebulic myrobalans have been included in the *materia medica* of Useibiah and others, indicates how much Arabic medicine was influenced by the Hindu medicine (Ray, 1902).

POSITION OF HINDU MEDICINE IN MEDIEVAL INDIA

The vegetable drugs employed by the Hindu physician and contained in the various books extant at the period mentioned above ran into several thousands, but during the invasion of India by the Greeks, Scythians, Huns, and the Mohammedans successively, a good deal of the existing Ayurvedic literature was mutilated or lost. Already the Buddhistic doctrine of *Ahimsa* (Non-killing), which led people to consider touching and dissection of dead bodies as sinful, had resulted in a decline of surgery. Medicine, however, had maintained its progress during the Buddhistic period and a large number of vegetable drugs were added to the already extensive *materia medica*. By the eleventh century or so, decadence set in generally, in knowledge, learning, and practice of both medicine and surgery.

When the Mohammedan rule was established in India from the thirteenth century on, the Arabian or the medieval Greco-Arab system, also known as the "Unani-Tibb," became the state system of medication. This system was also fairly advanced for that period, so that the old Hindu system of treatment was pushed to the background. The Arabic system brought with it a rich store of its own *materia medica*, unknown to India. During the long period of Mohammedan rule (up to the seventeenth century), this system came in close contact with the Ayurvedic or the Hindu system of medicine, resulting in a great deal of intermingling.

DECLINE AND EVOLUTION OF THE INDIGENOUS DRUGS OF INDIA

Both the Ayurvedic and the Unani-Tibb systems gradually declined with the fall of the Moghul Empire, coinciding with the advent of the Europeans in India—first the Portuguese, then the French and last the English. When the British had established their rule, the Western system of treatment was introduced in India. This further threw the indigenous systems (Ayurvedic and Unani-Tibb) into the background; nonetheless, both the ancient systems have remained the main source of medical relief to the bulk of the Indian population up to the present.

The modern Western system brought with it its own *materia medica*. Many of the indigenous drugs, however, after some pharmacological, chemical, and clinical investigations, were included in the British Pharmacopœia, and some of these like Kurchi, Ispaghula, Chirata, and Kaladana were accepted by the B. P. 1914. In the "Indian Pharmacopœial List" which has been published under the authority of the Government of India, all these drugs have been given recognition and many more like Rauwolfia, Punarnava, Vasak, etc., have been included. This "List" is considered of the same authority as the supplements to the B. P. published in Canada, Australia, and other dominions.

The earliest attempts at reviving the Indian drugs were confined mainly to the collection of available information about various medicinal plants. Sir William Jones' "Botanical Observations on Select Indian Plants" was one of the earliest contributions in this field (13). This was followed by John Fleming's "Catalogue of Medicinal Plants" (12), Ainslie's "Materia Medica of the Hindustan" (1), Roxburgh's "Flora Indica" (20), and later the works of Wallich (29), Royle (21, 23), and others (Strachey, 1852; Boissier, 1867; Stewart, 1874; Kurz, 1877; and Hooker 1875–97) (28, 2, 27, 15, 10). These did much toward resolving some degree of scientific arrangement from the chaos which existed in the vast mass of India's botanical material.

Of the earlier work on *materia medica* and the pharmacological aspect of Indian drug plants, mention may be made of O'Shaughnessy's "Bengal Pharmacopœia" (18), Irvine's "Materia

Medica of Patna" (11), "Pharmacopœia of India" by Dr. Waring (30), Dr. Mohideen Sheriff's "Supplement to the Pharmacopœia of India" (25), "Pharmacographia" by Flueckiger and Hanbury (9), Dutta's "Materia Medica of the Hindus" (6), and Dymock's "Vegetable Materia Medica of Western India" (7). But the two comprehensive works, namely, "Pharmacographia Indica" by Dymock, Warden, and Hooper (8) and "Dictionary of Economic Products of India" by Sir George Watt (31) are by far the most valuable of all the previous works and remain, with few exceptions, even today the final source for scientific reference. Works published later such as Kanny Lal Dey's "Indigenous Drugs of India" (5) and Kirtikar and Basu's "Indian Medicinal Plants" (14) are largely compilations from the last-named literature.

"Pharmacographia" by Flueckiger and Hanbury deals with vegetable drugs in England and in British India, while "Pharmacographia Indica" by Dymock, Warden, and Hooper is the pioneer work on the chemical aspect of Indian vegetable drugs. The Indigenous Drugs Committee (1901–1916) also did useful work in this line.

A perusal of "Indian Medicinal Plants" by Kirtikar and Basu, which is in four volumes and is provided with well-illustrated plates, testifies to the fact that a very large number of medicinal plants employed in India grow in different parts of the country. Indeed it has been said that about 2500 plants having alleged medicinal properties flourish there; these include about 300 plants mentioned in the British Pharmacopœia and the British Pharmaceutical Codex (3). Moreover, due to varied climatic and soil conditions India has the potentiality of producing exotic drugs, ranging from those growing in the hottest, tropical, and damp climate to those thriving in the dry, temperate, and cold regions. Cinchona, ipecac, digitalis, and pyrethrum may be taken as examples. However, as revealed by Chopra and co-workers (4), not many of the numerous indigenous drugs can be said to have any marked physiological activity. Much professionalism and orthodox belief in the curative properties of the indigenous drugs exist. A long and thorough scientific investigation is essential for sifting out the potent drugs from those devoid of any marked activity.

Of late years, a spirit of inquiry and research into the ancient systems has been manifested among the people. Detailed chemical and pharmacological investigations have been undertaken by some of the research institutes and medical colleges, notably the School of Tropical Medicine, Calcutta; the Haffkine Institute, Bombay; the Research Institute, Medical College, Madras; and the University of Travancore Research Institute. No doubt, with careful pharmacological, chemical, and pharmacognostical studies, claims concerning quite a number of the ancient Indian drugs will be justified.

There is a large group of people in India who still favor the revival of their indigenous systems of medicine. They argue that apart from economic considerations, these drugs are suited to the habits of the people and the climatic conditions that prevail in the country. In spite of a very strong reaction and inclination in this direction, there seems to be a clear understanding that medicine is universal. Hence it is to be hoped that before long the evolution now going on in India will lead to results useful to the Western as well as to the Indian people.

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Derivatives of Benzoic Acid and Simple Phenols in the Chemotherapy of Tuberculosis*,†

By F. A. FRENCH and B. L. FREEDLANDER

Bacteriostatic values of certain selected simple phenols, derivatives of benzoic acid and of salicylic acid, coumarins, and benzophenones against *M. tuberculosis* have been determined and the results are reported. Certain of these agents were also tested against other microorganisms, and *in vivo* in guinea pigs infected with *M. tuberculosis*, using streptomycin and *p*-aminosalicylic acid as reference drugs. A theory of the mechanism of action of salicylic acid and some of its derivatives is proposed.

THE GENERAL interest in aromatic acids as possible tuberculostatic chemotherapeutic agents rests on certain primary findings. Saz and Bernheim (3, 6), Bernheim (5, 9), and Fitzgerald and Bernheim (10) found that the oxygen uptake of *M. tuberculosis* strains H37Rv and B was stimulated greatly by salicylic acid while *M. tuberculosis* strain No. 607 was unaffected. The effect was apparently catalytic and the compound was not grossly metabolized. They found the meta and para isomers were not stimulatory to the pathogenic strains; however, recent investigations disclosed that all three compounds (Table I, Nos. 2, 3, 4) were mildly bacteriostatic. Fitzgerald and Bernheim (10) also found that cultures of *M. tuberculosis* H37Rv, adapted to growth in a salicylate concentration of 25 mg. per cent, were changed microscopically, culturally, and manifested increased virulence. In addition the adapted strain was less sensitive to *p*-aminosalicylic acid (PAS) inhibition.

These observations are of great interest and fundamental importance. Structural modifications of salicylic acid have led to the development of one agent, PAS (Lehman 12), of value in experimental tuberculosis and of definite clinical interest. In addition the present work and the work of Saz and Bernheim and co-workers (3, 7, 11) have shown that many compounds related to salicylic acid are highly bacteriostatic and highly specific for virulent tubercle bacilli.

In the following experiments a group of simple, related benzoic acid and phenol derivatives have been evaluated for tuberculostatic action *in vitro*. Additional *in vitro* studies were conducted with *M. leprae*, *M. phlei*, *M. tuberculosis* No. 607, *Trichophyton gypseum*, and a monilia (*Candida crusei*). Several of the compounds studied were investigated for their *in vivo* therapeutic action in experimentally induced tuberculosis in guinea pigs. Some of the compounds and a number of related compounds have been studied by other investigators (1–12). In the following work an attempt is made to correlate the results obtained here with those of other authors.

METHODS

The bacteriostatic studies were performed on virulent human tubercle bacilli strain No. H37Rv in Proskauer and Beck synthetic glycerin and asparagin media at a *p*H of 7.5. The chemicals were dissolved with a minimum of heating in propylene glycol or in 0.8% sodium hydroxide solution to make 0.5% solutions. Subsequent dilutions were made directly into the media without further heating. Each tube was inoculated with one loopful (4 mm. in diameter) of a fourteen-day-old surface culture of tubercle bacilli. These surface cultures were read at eighteen days using the highest dilution in which there was less than 50% of control growth as an end point.

Some additional tests were conducted with a few selected chemicals on *M. tuberculosis* No. 607 (a saprophytic mycobacterium), *M. phlei*, *M. leprae*, a monilia (*Candida crusei*), and *Trichophyton gypseum*. The methods of making dilutions, of inoculation, and of reading bacteriostatic end points were substantially the same as described for *M. tuberculosis* H37Rv. *M. leprae*, *M. phlei*, and *M. tuberculosis*, No. 607 were grown in Proskauer and Beck media at a *p*H of 7.2. They were incubated at 37° for five days, three days, and three days, respectively. *Trichophyton gypseum* was grown on Sabouraud's dextrose agar at a *p*H of 5.6 for five days at 22°; *Candida crusei* was grown on the same media at 37° for two days. In the case of the solid media the proper dilutions were made at 60° and the tubes were then slanted with no subsequent heating.

Animal Experiments.—Twelve guinea pigs weighing an average of 400 Gm. were used to evaluate each compound. The animals were inoculated in the left groin with 1.0 mg. wet weight of H37Rv tubercle bacilli. The drugs were administered once daily, orally by pipette, in aqueous suspension buffered to neutrality by sodium bicarbonate. Therapy was started on the day of infection. The dosage of all drugs was increased, gradually, for the first two weeks to the approximate chronic maximum tolerated dose; thereafter the dosage was reduced by 20%. The experiments were terminated at the end of six weeks. Pathology was evaluated numerically, based on a maximum of four for each organ (glands, spleen, liver, and lungs). Hemoglobin

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and weight determinations were made at the end of the experiments.

RESULTS

The *in vitro* bacteriostatic values are recorded in Table I. These values are selected from several experiments and may be considered to have a probable accuracy of ±1 dilution under the test conditions. The most extensively investigated compounds were the 4-ethers of 4-hydroxy and 2,4-dihydroxy benzoic acids. A gradual rise in bacteriostatic activity with increasing molecular weight of the ether group was noted in both series and the values were highest in the amyl ethers (compounds 13, 14, and 20). The simple phenols (compounds 26–30, inclusive) were mildly inhibitory. The compounds were bacteriostatic rather than bactericidal. Compounds 12, 19, 21, and 25 were tested against *M. leprae* and found inactive at a concentration of 10 mg. % while compound 30 just inhibited at 10 mg. %. Compound 15 was inactive against *M. phlei* at 0 mg. %. The monilia and *Trichophyton* tested were unaffected by 10 mg. % concentrations of compounds 19, 22, and 30. Compound 12 inhibited the *Trichophyton at 2.5 mg. %.*

The *in vivo* results are summarized in Table II. With the possible exception of compounds 33 and 35 which are similar structurally and gave low positive results, all compounds tested, other than the reference drugs (compounds 36 and 37), were devoid of *in vivo* activity. The value of these results consequently rests only in their correlative and extrapolative significance.

DISCUSSION

Our first interest in these simple aromatic compounds as tuberculostatic agents stemmed from consideration of the observation by Anderson and Newman (13) that *p*-anisic acid is a constituent of the lipoid fraction of virulent tubercle bacilli. We noted that it was not bacteriostatic and seemed to stimulate growth somewhat. The possible significance of this compound in the metabolism of tubercle bacilli appears to have been completely ignored. Following theoretical considerations closely analogous to those of Buu-Hoi (14), we undertook the synthesis and testing of a number of compounds bearing close structural relationships to *p*-anisic acid. When the methyl group was replaced by higher alkyl groups (compounds 10–13), inhibitors resulted. Similarly the *p*-alkyl ethers of 2,4-dihydroxybenzoic acid inhibited at almost exactly the same concentrations. These derivatives of 2,4-dihydroxybenzoic acid may be regarded as close polar analogs of PAS but their level of inhibition and quite possibly their mechanism of action is very different. It is interesting to note that additional nuclear substitution gave decreased bacteriostatic activity.

Umbelliferone and β-methylumbelliferone were included because of their vinylogous relation to 2,4-dihydroxybenzoic acid. Compound 33 can be regarded as a vinylog of salicylic acid (No. 2); compound 34 as a vinylog of *p*-hydroxybenzoic acid (No. 4); and compound 35 as a vinylog of 3,5-dichlorobenzoic acid. These benzophenones (compounds 33–35) possess the same type of hydrogen bond ring structure found in salicylic acid. The fact that activities are maintained or even increased in the vinylogs is encouraging and points to certain possible further extensions in this group of drugs. The principle of vinylogy may apply in these cases because the critical spacings between polar centers are not grossly changed.

No adequate correlative interpretation of the antituberculous activity of simple salicylic acid

TABLE I.—BACTERIOSTATIC ACTION ON MYCOBACTERIUM TUBERCULOSIS STRAINS H37Rv AND 607[a]

No.	Compound	Min. Inhib. Conc. Mg. %[b] H37Rv	607
1	Benzoic Acid	5.	...
2	2-Hydroxybenzoic Acid	2.5	50
3	3-Hydroxybenzoic Acid	5.	50
4	4-Hydroxybenzoic Acid	2.5	50
5	2-Methoxybenzoic Acid	10.	50
6	3-Methoxybenzoic Acid	10.	>50
7	4-Methoxybenzoic Acid	>10.	>100
8	4-Methoxy-3-aminobenzoic Acid	10.	100
9	4-Methoxy-3,5-dichlorobenzoic Acid	>10.	50
10	4-Ethoxybenzoic Acid	2.5	100
11	4-*n*-Propoxybenzoic Acid	1.	...
12	4-*n*-Butoxybenzoic Acid	0.25	...
13	4-*n*-Amoxybenzoic Acid	0.025	50
14	3-*n*-Amoxybenzoic Acid	0.025	...
15	4-Methoxy-2-hydroxybenzoic Acid	2.5	...
16	4-Ethoxy-2-hydroxybenzoic Acid	2.5	>10
17	4-*n*-Propoxy-2-hydroxybenzoic Acid	1.	...
18	4-Isopropoxy-2-hydroxybenzoic Acid	1.	...
19	4-*n*-Butoxy-2-hydroxybenzoic Acid	0.25	50
20	4-*n*-Amoxy-2-hydroxybenzoic Acid	0.025	...
21	2,4-Dihydroxybenzoic Acid	1.	...
22	2,4-Diacetoxybenzoic Acid	1.	...
23	2,4-Dihydroxy-5-chlorobenzoic Acid	>10.	...
24	4-Ethoxy-5-chloro-2-hydroxybenzoic Acid	2.5	...
25	Orsellinic Acid	10.	...
26	Phenol	5.	...
27	Catechol	2.5	...
28	Resorcinol	10.	...
29	Hydroquinone	5.	...
30	Orcinol	1.	10
31	Umbelliferone	1.	...
32	β-Methylumbelliferone	0.25	...
33	2,2′-Dihydroxybenzophenone	1.	...
34	2,4′-Dihydroxybenzophenone	1.	...
35	2-Hydroxy-3,5-dichlorobenzophenone	0.25	...
36	*p*-Aminosalicylic Acid[c]	0.0025	...
37	Streptomycin[c]	0.001	...

[a] The compounds in this table were prepared in this laboratory under the direction of F. A. French with the following exceptions: Nos. 1–7 and 26–30, inclusive, were obtained from the Eastman Kodak Co. (highest purity); Nos. [illegible] the Koppers Co.; No. 35 was [illegible] Chemical Co.; No. 36 by the [illegible] by E. R. Squibb and Sons.

[b] These are the minimum concentrations giving greater than 50% inhibition of bacterial growth.

[c] *p*-Aminosalicylic acid and streptomycin were included for purposes of comparison.

TABLE II.—SUMMARY OF *in Vivo* EXPERIMENTAL DATA

No.	Compound	Av. Daily Dosage, Mg. per Animal	Path. Ratio[a]	Relative Wt. Gain, Gm. Deviation from Controls	Hemoglobin % Deviation from Controls
8	4-Methoxy-3-aminobenzoic Acid	90	1.16	−6	0
9	4-Methoxy-3,5-dichlorobenzoic Acid	151	1.02	−35	+2
13	4-*n*-Amoxybenzoic Acid	60	1.14	+13	−2
15	4-Methoxy-2-hydroxybenzoic Acid	122	0.96	0	−11
16	4-Ethoxy-2-hydroxybenzoic Acid	151	1.10	−26	−10
17	4-*n*-Propoxy-2-hydroxybenzoic Acid	134	1.02	−5	−3
18	4-Isopropoxy-2-hydroxybenzoic Acid	127	1.10	−19	−2
19	4-*n*-Butoxy-2-hydroxybenzoic Acid	64	1.01	−52	−10
22	2,4-Diacetoxybenzoic Acid	134	1.01	−33	−3
24	4-Ethoxy-5-chloro-2-hydroxybenzoic Acid	103	1.07	−65	0
32	β-Methylumbelliferone	150	1.04	−43	−3
33	2,2′-Dihydroxybenzophenone	141	1.22	−1	+2
35	2-Hydroxy-3,5-dichlorobenzophenone	153	1.25	−59	−2
36	*p*-Aminosalicylic Acid	175	1.60	+111	−11
37	Streptomycin[b]	20 (hypo)	3.90	+123	+8.5

[a] Pathological ratio is the quotient of the average numerical pathological rating of the control animals divided by that of the treated animals.
[b] 40 mg. of streptomycin was given by hypo every two days.

derivatives has as yet been published. If it is borne in mind that, in animals, a portion of administered salicylate is converted to gentisic acid (15) and that gentisic acid has recently been used successfully for symptomatic treatment of rheumatic fever (16) some tentative hypotheses can be formulated. Gentisic acid is capable of entering into rapid-reversible oxidation-reduction reactions. In its oxidized (quinone) form it is also known to inactivate hyaluronidase *in vitro* (17). It is quite conceivable, then, that gentisic acid could accelerate certain oxidative metabolism and interfere with certain growth processes in tubercle bacilli. Similarly, some oxidized form of PAS such as 4-amino-2,5-dihydroxybenzoic acid or a further oxidation product could be, through its high and specific combining power, responsible for its bacteriostatic action. It is well known that many compounds which are capable of entering into para-quinoid oxidation-reduction systems are powerful and frequently very selective inhibitors of virulent tubercle bacilli. Examples include the para-alkoxy-benzenes and heterocycles studied by Feinstone, Friedman, and co-workers (18) and the 6-amino-2-substituted benzothiazoles studied by the present authors (19). It would seem then that virulent tubercle bacilli contain a system or systems which can interact, to the detriment of the organism, with many compounds which can form paraquinoid oxidation-reduction systems. The character of the additional substituents and their positionality is critically important. One fundamental approach to the chemotherapy of tuberculosis lies in the further synthesis and trial of a great variety of compounds which can give rise to para-oxidation-reduction systems and which bear additional substituents in various type patterns. The major problems are maintaining and heightening activity and selectivity while attempting to develop drugs of better *in vivo* absorption-distribution-excretion characteristics.

The lack of *in vivo* activity among the compounds reported here is undoubtedly due to unfavorable absorption-distribution-excretion factors. The carboxyl group in a great number of aromatic acids is readily conjugated and several instances are known in which aromatic ethers are readily dealkylated and conjugated *in vivo* (15). Further studies are in progress to extend this group of drugs and to attempt to find agents with better *in vivo* characteristics.

SUMMARY

1. Bacteriostatic values against *M. tuberculosis* H37Rv were determined for five simple phenols, twenty-six derivatives of benzoic and salicylic acids, two coumarins, and three benzophenones. Selected agents were tested against *M. tuberculosis* No. 607, *M. leprae*, *M. phlei*, a monilia, and *Trichophyton gypseum*.

2. Thirteen selected compounds were tested *in vivo* in guinea pigs infected with *M. tuberculosis* H37Rv using streptomycin and *p*-aminosalicylic acid as reference drugs.

3. Structure-bacteriostatic activity relations are analyzed and a theory of the mechanism of action of salicylic acid and some of its derivatives on tubercle bacilli is proposed.

4. Only two low *in vivo* activities were found. Further work is in progress.

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The Antiadrenolytic Action of Ergotamine*

By THEODORE O. KING† and THEODORE KOPPANYI

The vasomotor reversal of Dale is generally considered to be due to depression of the vasoconstrictor, but not of the vasodilator sympathetic neuro-effectors, leaving epinephrine free to stimulate the vasodilator, but not the vasoconstrictor sympathetc terminations. This phenomenon, called adrenolysis, may be caused by ergotamine, dibenamine, and other drugs. In dogs under nembutal anesthesia it was shown that ergotamine at certain dose levels not only does not reverse, but actually potentiates, the pressor effect of epinephrine. Moreover, when dibenamine and other powerful "sympatholytic" agents were used to produce vasomotor reversal, ergotamine in doses of 0.05 mg./Kg. or more restored the original pressor effect of epinephrine. Thus, ergotamine under certain experimental conditions reverses the effect of other adrenolytic drugs

IN DOGS, under pentobarbital anesthesia, ergotamine tartrate, in doses from 0.05 to 1.0 mg./Kg. or more, potentiates the pressor effects of epinephrine and also other sympathetic stimulants (2, 6). King (3) reported that ergotamine in similar doses converts the vasodepression produced by various doses of N-isopropyl-nor-epinephrine and N-isopropyl-nor-sympatol to marked pressor effects. These observations suggested the study of the effects of ergotamine on the vasomotor reversal of epinephrine induced by potent adrenolytic or sympatholytic agents in dogs anesthetized by sodium pentobarbital.

EXPERIMENTAL

Eleven animals were used for these experiments. At first the average hemodynamic responses were established by the intravenous injection of fixed amounts of epinephrine hydrochloride, nor-epinephrine hydrochloride, and of N-isopropyl-nor-epinephrine hydrochloride. Then the adrenolytic drug was injected either in a single dose or in divided doses by vein. In every case sufficient amounts were given to produce marked vasomotor reversal of epinephrine (criterion: the larger the dose of epinephrine, the greater the fall in blood pressure), to reduce or abolish the pressor effect of nor-epinephrine, and to increase the depressor effect of N-isopropyl-nor-epinephrine. Following the establishment of satisfactory vasomotor reversals, ergotamine tartrate was injected in doses of 0.05 mg./Kg., and in some cases this dose was increased to 0.2 mg./Kg. Five minutes after the administration of ergotamine, the injections of epinephrine, nor-epinephrine, and N-isopropyl-nor-epinephrine were repeated with the result that the epinephrine reversal was abolished and converted into a rise usually greater than the effect of the control injection. The nor-epinephrine pressor effects were also increased and the vasodepressor effects of N-isopropyl-nor-epinephrine were converted into marked pressor effects (Fig. 1). These pressor effects were not only sharp but of relatively long duration. Table I summarizes the effects of ergotamine on the vasomotor reversal and other effects produced by benzyl-imidazoline (Priscol), benzyl-β-chloroethylamine (Dibenamine), diethylaminomethylbenzodioxane (883 F), piperidinomethylbenzodioxane (933 F), and yohimbine hydrochloride.

The experiments were devised so as to exclude possible sources of error. In many cases the vasomotor reversing agent was administered and its effect tested. An hour later when vasomotor reversal was still present the full vasomotor reversing dose was again administered and only then was the effect of ergotamine studied on the responses to epinephrine, nor-epinephrine, and N-isopropyl-nor-epinephrine. This eliminated the possibility that ergotamine was counteracting the effect of a drug that is in part already essentially eliminated. The possibility that ergotamine produces vasospasm and thus generally

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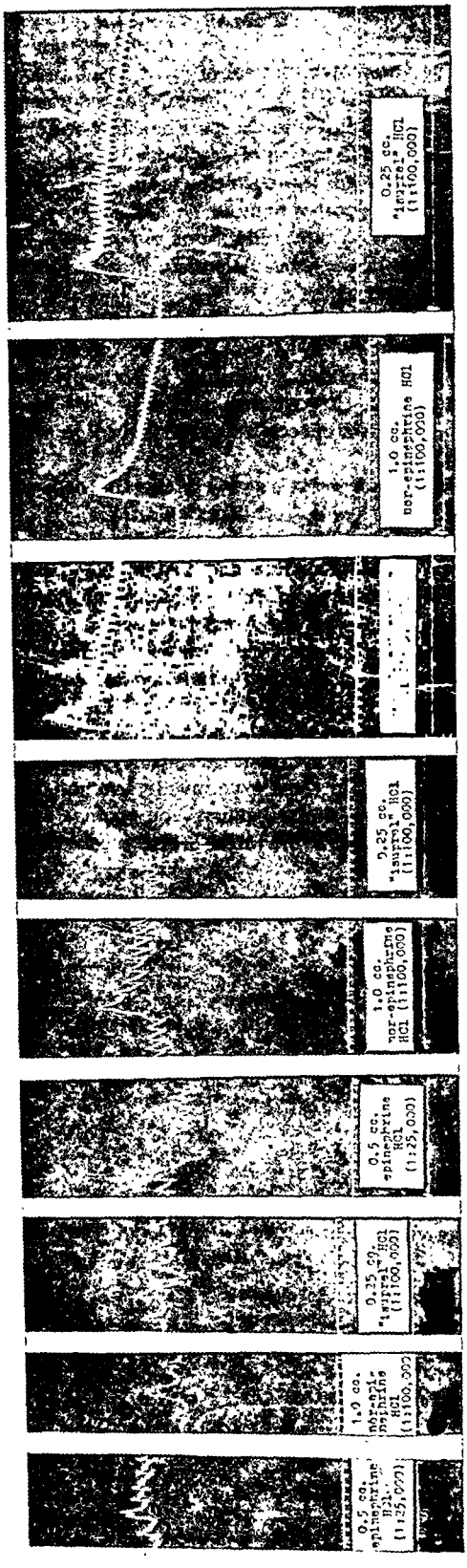

Fig. 1.—Dog, 16.6 Kg., 35 mg. of sodium pentobarbital per kilogram by vein. Upper line represents mean arterial blood pressure recorded from the common carotid artery; middle line (base line, representing zero mm. of Hg pressure)—time, 5 seconds; lower line indicates injection of drugs. *1*, 0.5 cc. epinephrine hydrochloride (1:25,000), vein. *2*, 1.0 cc. nor-epinephrine hydrochloride (1:100,000), vein. *3*, 0.25 cc. "isuprel" hydrochloride (1:100,000), vein; 6.0 mg. dibenamine hydrochloride per kilogram, vein, between 3 and *4*. *4*, 30 minutes later, same as in *1*. *5*, same as in *2*. *6*, same as in *3*; 0.05 mg. ergotamine tartrate per kilogram, vein, between 6 and 7. *7*, 30 minutes later, same as in *1*. *8*, same as in *2*. *9*, same as in *3*.

abolishes vasodilator or vasodepressor effects was investigated. It was, indeed, shown that the depressor effects of acetylcholine and histamine were appreciably reduced by ergotamine administration (cf. 7). On the other hand, when vasospasm was produced by barium chloride (1.0 mg./Kg.) (cf. 5) or by Pitressin (cf. 4), the extent of vasodepression of epinephrine and N-isopropyl-nor-epinephrine was somewhat reduced but never completely abolished or reversed. Finally, when sodium nitrite (10 mg./Kg.) was administered intravenously the ergotamine effect on the vasomotor reversal and on N-isopropyl-nor-epinephrine vasodepression was preserved in spite of the profound general vasodilation. The duration of action of ergotamine in abolishing the vasomotor reversal is very prolonged and lasts for several hours. By this time the vasospasm, at least judged by the return of blood pressure to normal or subnormal levels, appeared to have diminished or disappeared.

DISCUSSION

Ahlquist, *et al.* (1), reported an antiadrenolytic action of ephedrine which caused the pressor effect of epinephrine to reappear after it had been blocked by Priscol. Their tracing indicates that Priscol did not reverse the pressor effect of 5γ of epinephrine but merely abolished it, and that following the administration of 5 mg. ephedrine, the same dose of epinephrine produced a rise smaller than the original. The most plausible explanations they offer for these phenomena are the resensitization of the pressor receptors by ephedrine or the blocking of the epinephrine depressor mechanism. Shaw and MacCallum (8) report that in cats, curare possesses the power of reversing the vasomotor reversing action of yohimbine. They offer the explanation that curare antagonizes yohimbine or that curare potentiates only the excitor or constrictor action of epinephrine and may antagonize the depressor or dilator action.

Apart from these isolated observations, the experiments reported above offer the first systematic proof that the vasomotor reversal caused by any type of adrenolytic or sympatholytic agent can be instantly reversed by another drug and, curiously, by a drug known to possess, under certain conditions, adrenolytic properties (2). The expected result should be an additive synergism and not an antagonism. Moreover, one can hardly escape the conclusion that unless there is a chemical antagonism between ergotamine and the other adrenolytic agents—and this assumption is hardly warranted—the adrenolytic and sympatholytic actions of dibenamine and other compounds, at least in the concentrations employed, are apparent rather than real. Furthermore, the experiments

TABLE I.—AVERAGE HEMODYNAMIC RESPONSES TO EPINEPHRINE, NOR-EPINEPHRINE, AND ISOPROPYL-NOR-EPINEPHRINE FOLLOWING THE ADMINISTRATION OF ADRENOLYTIC AGENTS AND ERGOTAMINE

	Blood-Pressure Changes in Mm. of Hg[a]		
	Control	After Adrenolytic	After Ergotamine (0.05–2.0 Mg./Kg.)
883 F, 6.0 Mg./Kg. (3 dogs)			
Epinephrine[b]	18	−18[a]	30
Nor-epinephrine[b]	26	12	44
N-Isopropyl-nor-epinephrine[b]	−15	−36	32
933 F, 12.0 Mg./Kg. (2 dogs)			
Epinephrine	17	−12	36
Nor-epinephrine	28	12	44
N-Isopropyl-nor-epinephrine	−32	−45	36
Dibenamine HCl, 6.0–10.0 Mg./Kg. (2 dogs)			
Epinephrine	15	−22	56
Nor-epinephrine	26	13	44
N-Isopropyl-nor-epinephrine	−14	−32	46
Priscol HCl, 7.0 Mg./Kg (2 dogs)			
Epinephrine	27	−9	35
Nor-epinephrine	30	8	40
N-Isopropyl-nor-epinephrine	−23	−20	56
Yohimbine HCl, 7.0 Mg./Kg. (2 dogs)			
Epinephrine	24	−10	22
Nor-epinephrine	18	6	46
N-Isopropyl-nor-epinephrine	3, −12	−18	34

[a] Figures marked "−" indicate vasodepression
[b] All doses of epinephrine, nor-epinephrine, and isopropyl-nor-epinephrine, 0.2 γ/Kg.

strongly suggest that the so-called adrenolytic drugs do not paralyze the excitor or constrictor sympathetic vasomotor receptors, but rather sensitize the depressor or dilator sympathetic or other receptors. This is particularly shown by the parallelism of the effects of the five adrenolytic agents and ergotamine on the effects of epinephrine and N-isopropyl-nor-epinephrine. In both cases it appears that the vasodepressor mechanism is sensitized by the five sympatholytic agents and desensitized by ergotamine. It is admitted that a part of this desensitization, at least during its initial phase, is due to the general vasospasm produced by ergotamine.

SUMMARY

The vasomotor reversal of epinephrine caused by Priscol, 883 F, 933 F, dibenamine, and yohimbine is abolished and the pressor effect restored by the administration of ergotamine.

Nor-epinephrine pressor effects are not reversed but merely greatly reduced or abolished by the adrenolytic agents. Ergotamine not only restores but exaggerates the pressor effects over the normal values.

In general, Priscol, 883 F, 933 F, dibenamine, and yohimbine, in adrenolytic doses, accentuate the vasodepression caused by N isopropyl-nor-epinephrine. Ergotamine not only abolishes these depressor effects but converts them to pressor effects.

The antiadrenolytic action of effective doses of ergotamine is of several hours' duration.

Ergotamine also antagonizes the depressor effects of acetylcholine and histamine. Drugs producing vasospasm, such as barium or Pitressin, do not possess antiadrenolytic action.

The antiadrenolytic action of ergotamine persists even when the vasospasm is counteracted by nitrites.

It is suggested that the adrenolytic effects of Priscol, 883 F, 933 F, dibenamine, and yohimbine, in doses used in these experiments, are not due to paralysis of motor sympathetic endings, but to a sensitization of the vasodepressor mechanism.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Indican
Coriamyrtin
L-Lyxose
9-Methyl-2,6,7-trihydroxy-3-fluorone
9-Phenyl-2,6,7-trihydroxy-3-fluorone
Phenyl pantothenone
4′-Methyl-3,4-benzepyrene
20-*tert*-Butylcholanthrene
15,20-Dimethylcholanthrene
20-Ethylcholanthrene
5-Nitro-3,4-benzpyrene
3-Chloro-20-methylcholanthrene
6-Cyano-20-methylcholanthrene
D-5-Ketogluconic acid
Hemocyanin
3,7,2′,4′-Tetrahydroxyflavone
3,7,8,2′-Tetrahydroxyflavone
1-Nitroacridine
1-Hydroxy-2-anthramine
1,6-Dihydroxynaphthalene
Sinigrin
Hemopyrrole

The Antipyretic Action of Some Derivatives of Salicylic Acid, *p*-Aminophenol, and Dihydropyrazolon*

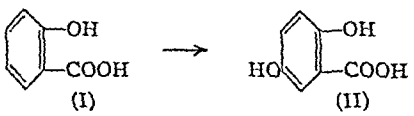
By C. I. JAROWSKI† and M. K. McPHAIL‡,§

The antipyretic actions in rabbits of six salicylate derivatives, an addition product of carbomethoxybenzoquinone and methyl gentisate have been determined and are reported. None of the compounds studied was significantly effective in lowering the temperature of the animals.

It is not known at the present time whether the action of drugs used as analgesics and antipyretics in rheumatic fever therapy results from the compounds *per se* or from some metabolites derived from their degradation. In the present study a number of compounds was prepared and evaluated for their ability to lower the temperature of rabbits made febrile by injection of a suspension of *Proteus vulgaris*.

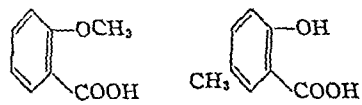
The various compounds studied can be considered as belonging to one, and in some cases to two, of the following classes into which the analgesic-antipyretic drugs are usually divided (1): (I) the salicylate-cinchophen group; (II) the *p*-aminophenol derivatives; (III) the pyrazolon derivatives. The compounds belonging to the first two classes will be discussed under a single heading because of their structural relationship.

The Salicylate-p-Aminophenol Group.—The desirability of testing a metabolite of salicylic acid was indicated by a number of observations. Kapp and Coburn (2) showed that salicyluric, gentisic, salicylglucoronic, and salicylic acids were present in the urine of patients given sodium salicylate. Patients with acute rheumatic fever excrete less salicyl than normal subjects, but the gentisic acid concentration of the former group is two to four times greater than that found in healthy subjects. This finding suggests that the first step in the oxidative degradation of salicylic acid (I) is its conversion to gentisic acid (II).



(I) Salicylic Acid → (II) Gentisic Acid

The decreased excretion of salicyl in the rheumatic patient suggests that further degradation of gentisic acid occurs. Thus one might infer that the active therapeutic agent in alleviating rheumatic symptoms is either gentisic acid or one or more of its degradation products, a view which receives support from the recent findings of Meyer and Ragan (3) that sodium gentisate possesses antirheumatic activity equal to, or greater than, that of salicylate. Indirect evidence for this assumption is also suggested by the fact that the methyl ether of salicylic acid (III) (4) and 5-methyl salicylic acid (IV) (5) are less effective antirheumatic drugs, and neither compound could easily be converted to gentisic acid under biological conditions.



(III) Methyl Ether of Salicylic Acid (IV) 5-Methyl Salicylic Acid

Likewise neither meta- nor para-hydroxy benzoic acids are effective antirheumatic agents (5). It is to be noted that the carboxyl group in the former compound would inhibit its conversion to gentisic acid since its meta-orienting influence would tend to inactivate the position para to the phenolic group. Thus there is some indirect evidence which appears to indicate that salicylate compounds which cannot easily be converted to gentisic acid do not possess good antirheumatic properties.

In addition to studying the action of gentisic acid for the reasons mentioned above, 5-aminosalicylic acid, 5-amino ethyl salicylate hydrobromide, and 5-amino methyl salicylate hydrobromide were included because it was of interest to determine the activity of compounds which could be considered as *p*-aminophenol as well as salicylic acid type compounds. These substances could also be degraded in the same manner as gentisic acid under biological conditions.

Since it was found that mild oxidation of methyl gentisate converts it to carbomethoxybenzoquinone (V), which in the presence of excess methyl gentisate forms an addition compound (VI), it was also believed of interest to test the addition compound. (Carbomethoxy-benzoquinone (V) was not isolated and hence could not be tested.)

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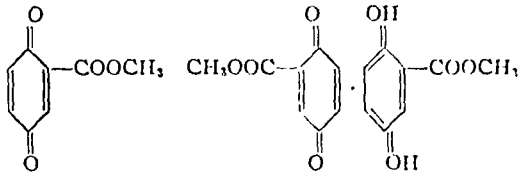
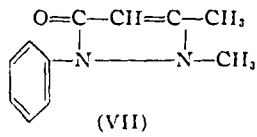
§ The authors are indebted to Mr. T. Wombolt for his technical assistance.



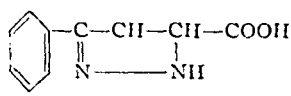
(V) (VI)

In addition to the above compounds salicyl uric acid, a normal excretory product of patients receiving salicylate, was tested. Methyl gentisate was tested because of the possibility of its being less rapidly excreted and as a consequence more effective than gentisic acid.

The Pyrazolon Derivatives.—Since antipyrine (VII) is also an analgesic-antipyretic derivative, a dihydropyrazolon derivative (VIII) was prepared to determine its activity. It will be noted that in this derivative the phenyl group is attached to carbon in the heterocyclic ring while in antipyrine it is attached to nitrogen.



(VII)



(VIII)

Aspirin and amidopyrine were included in the tests, since aspirin is a representative of the salicylate-cinchophen group and amidopyrine is an example of the *p*-aminophenol derivatives.

EXPERIMENTAL

Preparation of Compounds.—The following compounds were prepared by methods described in the literature: gentisic acid (6), addition product of carbomethoxybenzoquinone and methyl gentisate (7), 2-carboxy-5-phenyl-dihydropyrazolon (8), salicyluric acid (9), and 5-amino salicylic acid (10).

Preparation of Pyrogen.—*Proteus vulgaris* was grown on nutrient agar for twenty-four hours at 37°. The growth was washed off in physiological saline and the concentration of organisms standardized to contain 10 billion per cc. This stock suspension was preserved with 0.5% phenol and stored at 4°. For the actual test, the stock suspension was diluted with sterile saline to contain approximately 2×10^7 organisms per cc., and one cc. of this suspension was injected into the marginal ear vein of rabbits to produce pyresis. This dose caused a 1° to 2° rise in temperature which lasted two to four hours.

Test Procedure.—All drugs were given orally suspended in 10% gum acacia, except where otherwise noted. In most cases 3 animals were given the drug and 3 were given the acacia suspension as controls for each test. In conducting the tests th rabbits were immobilized in stocks (11) and the rectal temperatures recorded (with a Centigrac thermometer graduated in 0.1° units) for 2 succe sive periods, thirty minutes apart. Then the pyr gen and drug were administered and the rectal tem peratures recorded every half hour for the follo ing five or six hours while the animals remained the stocks. Since Beeson (12) has reported th rabbits injected daily with pyrogenic material show progressive diminution in febrile response, it w believed important to avoid such a possibility this in our work and therefore the animals we given rest periods of several days to a week b tween tests.

RESULTS

Statistical Treatment.—The data were treated i the following manner: The temperature departur (plus or minus) from the initial or zero temperatur averaged over the test period of five to six hour were tabulated for all animals of the experiment group and compared with the same figures for t control group. Since the only distinction betwe the two was that the first received the test dr and the second an inert suspension, then the di ference between them should express the antipyret potency of the test compound. The analysis w made by using "Student's" *t* test which examin the probability that the differences found betwe experimental and control groups can be account for on the basis of random sampling alone. T average temperature departures from the norm for the two groups for all compounds tested are give in Table I, along with the *t* values, the probabili *p*, and the significance. Probability values 0.05 or less were considered to be significant.

Graphs.—In addition to the statistical analysi graphs were prepared for each drug tested showin the average initial temperature, considered as zer and the average rise or fall (plus or minus) from th initial temperature at thirty-minute intervals durin the succeeding five or six hours for both the exper mental and control groups of animals. Exampl of the types of graph prepared are shown in Figs. and 2. Figure 1 is shown to demonstrate the degre of pyrexia produced, and the antipyretic action amidopyrine. Figure 2 represents the negativ type of response found with all of the com pounds tested, with the exception of amido pyrine.

DISCUSSION AND CONCLUSIONS

It is evident from an examination of Table that the only drug studied which showed a statis tically significant antipyretic action was amidopyrin when administered in a dose of 200 mg./Kg. (Ex periments 9 and 10). When this compound wa given in a dose of 100 mg./Kg. (Experiment 15 the result was not statistically significant althoug antipyresis occurred as was evident from the grap (not shown) and the average temperature change of the experimental and control groups (column 6 and 7). Acetylsalicylic acid did not have a sta tistically significant antipyretic action althoug in the two experiments in which it was given th treated animals had slightly lower temperature than their controls. 5-Amino methyl salicylat

TABLE I.—ANTIPYRETIC ACTIVITY OF SOME DERIVATIVES OF SALICYLIC ACID, *p*-AMINOPHENOL, AND DIHYDROPYRAZOLON

Expt. No.	Compound	Dose Mg./Kg.	No. Experimental Animals	No. Control Animals	Average Temperature Departures from Normal (°C). Experimental Animals	Control Animals	*t* Value	*p* Value	Significance[a]
1	Gentisic Acid	200	1	3	+0.57[c]	+1.01	1.20	0.3	N. S.
2	Gentisic Acid (Na Salt)[b]	100	1	..					
3	Gentisic Acid (Na Salt)[b]	222.5	1	3					
4	5-Aminosalicylic Acid	250	4	2	+0.67	+0.66	0.06	0.9	N. S.
5	5-Amino Ethyl Salicylate HBr	500	4	2	+0.57	+0.65	0.54	0.6	N. S.
6	5-Aminosalicylic Acid	250	3	3	+1.18	+1.08	0.27	0.8	N. S.
7	Acetylsalicylic Acid	250	3	3	+1.13	+1.32	0.73	0.5	N. S.
8	Acetylsalicylic Acid	250	3	3	+1.09	+1.28	0.65	0.6	N. S.
9	Amidopyrine	200	3	3	−0.24	+0.98	3.56	0.02	S.
10	Amidopyrine	200	3	3	+0.12	+1.38	4.70	0.01	S.
11	5-Amino Methyl Salicylate HBr	250	3	3	+0.43	+0.24	0.39	0.7	N. S.
12	Methyl Gentisate	250	2	3	+1.05	+0.78	1.48	0.25	N. S.
13	Dihydropyrazolon Der. (VIII)	200	3	3	+1.24	+1.33	0.39	0.7	N. S
14	Addition Product (VI)	100	2	2	+0.42	+0.33	0.32	0.8	N. S.
15	Amidopyrine	100	2	2	+0.06	+0.33	1.59	0.25	N. S
16	Salicyluric Acid	250	3	3	+0.66	+0.63	0.07	0.9	N. S.

a
b
c

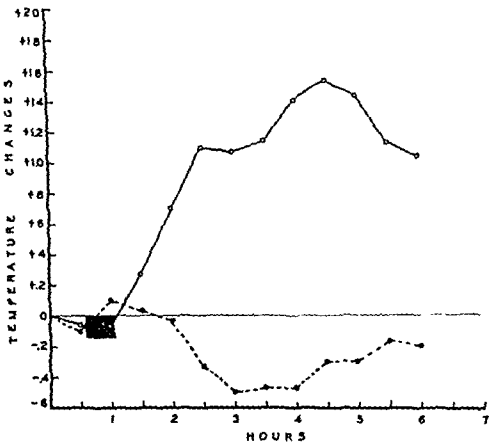


Fig. 1.—The lower curve (broken line) illustrates the average temperature changes of 3 rabbits given 200 mg./Kg. of amidopyrine in 10% gum acacia; the upper curve (solid line), the temperature changes of 3 control rabbits giving acacia only. The hatched area indicates the period of drug administration. Amidopyrine caused a fall in temperature well below the initial temperature; the pyrogen, an increase in temperature of about 1.5°.

hydrobromide caused a slight elevation in temperature and methyl gentisate a somewhat greater rise. Neither of these differences, however, proved to be statistically significant and the drugs were not investigated further. Rabbits given gentisic acid had lower temperatures than the controls but the difference between the two groups was not found to be significant. Unfortunately it was possible to administer gentisic acid only to a single rabbit on each of three experiments and the test probably suffers as a result.

The drugs which we have studied belong to either the salicylate-cinchophen, *p*-aminophenol, or pyrazolon group, members of which generally are considered to possess both analgesic and antipyretic activity. As none of the compounds tested showed marked antipyretic powers, it was assumed, when the work was first undertaken, that they would possess no important analgesic action and would, therefore, be of little value in the treatment of rheumatic fever. In view of Meyer and Ragan's recent report (3), however, on the antirheumatic action of sodium gentisate it would appear that this assumption is not tenable and that the antipyretic action

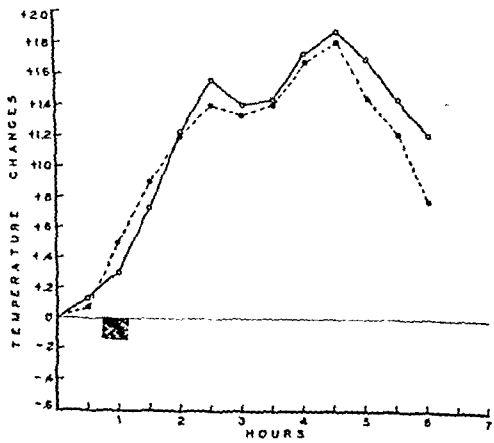


Fig. 2.—The broken line illustrates the average temperature changes of 3 rabbits given 200 mg./Kg. of the dihydropyrazolon derivative VIII in 10% gum acacia; the solid line, the temperature changes of 3 rabbits given acacia only. The hatched area indicates the period of drug administration. The ineffectiveness of compound VIII is apparent.

of a compound cannot be used as a criterion for its activity in the rheumatic state. In other words, the change of pain threshold cannot be measured by the change of heat threshold.

SUMMARY

Six salicylate derivatives, 3 pyrazoline compounds, and an addition product of carbomethozy-benzoquinone and methyl gentisate were tested for their antipyretic action in rabbits. None of these compounds was effective in lowering the temperature of the animals to a significant degree.

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By ALFRED HALPERN† and JAMES W. JONES‡

Based on experimental data involving the preparation of trialkylbarbituric acid derivatives and studies of degradative products of these compounds, a dynamic ionic structure for metallic derivatives of the barbiturates is proposed.

THE DYNAMIC PROPERTY of tautomerism has been consistently applied to the behavior of barbituric acid and its derivatives, yet the structure commonly used to designate the metallic derivatives is the static enol form. A review of the literature revealed many marked differences of opinion even to this static structure, with some authors favoring the keto-lactam form while others prefer the enol-lactim structure. Beilstein (1) refers to the sodium salt of barbital as the nitrogen derivative "or its desmotrope." The U. S. P. XIII and Merck's Index designate this structure as the enol.

There is a conspicuous absence in the literature of a 2,4- or 6-alkyl derivative resulting from the direct alkylation of the metal barbiturate with an alkyl halide. The products obtained from this reaction have been shown to be the 1,3- or 5- substituted compounds. Marotta and Rosanova (2) reported that they had obtained the enol ether as a result of the treatment of barbital with diazomethane. Their tautomeric product was subsequently shown to be identical with 1,3-dimethyl-5,5-diethylbarbituric acid prepared by Dox (3). The existence of the ether analogs has been demonstrated (4, 5) but these were not obtained by the direct alkylation of a barbiturate, rather through the reaction between the tetrachlorodihydropyrimidine and sodium alkoxide. The tetrachlorodihydropyrimidine, however, was prepared by treating barbital with phosphorous pentachloride and then converted to the tautomer of tetraethylbarbituric acid through alkylation with sodium ethoxide (5). This compound is quite stable and is not prone to rearrange. From analogy to related compounds, as isatin and carbostyril, the tautomeric enol form should be the less stable one, nevertheless, the ether, once formed, should be relatively stable, although intramolecular rearrangement may occur. The theoretical existence of the tautomers is established from the knowledge of bond energies which indicate that the C—O bond is more stable than the C—N bond (6).

Two isomeric methyl derivatives of isatin are known. The ether is obtained by converting potassium isatin into the silver salt and heating with methyl iodide. The N-analog may be obtained by the action of sodium hypobromite on N-methylindole. The constitution of these compounds was further established by the isolation of *o*-aminophenylglyoxylic acid from the hydrolysis of the ether and *o*-methylaminophenylglyoxylic acid from similar treatment of the N-substituted derivative. It was of interest to determine whether the tautomer of 1- or 3-alkylbarbital

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could be prepared through the analogous use of silver.

Since three tautomeric structures can be postulated for the monometallic derivatives of barbital, it is possible that the product which is usually described as the 1- or 3-alkylbarbital, resulting from the direct alkylation procedures, was in reality an equilibrium mixture of the N-substituted derivative and its O-analog. For the most part the reaction products isolated from the direct alkylation procedures were not investigated further to determine whether the ether analog might be present. An elementary analysis would not distinguish between the two tautomers.

This study was initiated to determine the possibility of the ether forming as a result of a direct alkylation procedure leading to the formation of a trialkylbarbituric acid derivative.

EXPERIMENTAL

Method A.—N-alkylbarbital was prepared through the reaction between ethyl diethylmalonate and the appropriate N-alkyl urea in the presence of sodium methoxide (7). The yields, melting points, and analysis of the various products are given in Table I.

Method C.—Molar equivalents of sodium barbital and an alkyl halide and 0.5 Gm. of copper powder were stirred for thirty minutes in 500 cc. of water at room temperature. The separated material was treated as in Method B (9).

This reaction is exothermic and has the advantage that there is no formation of a dialkylurea as was observed from the prolonged boiling of a barbituric acid with alkali (5).

Method D.—This was identical with Method C except that 1.236 Gm. of copper sulfate was substituted for the copper powder. The copper concentrations in both methods was equivalent.

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E-ethanol	28	38	30	34	32	154	117	36	127	74–75	...	...	...	...	...
F	75	71	78	76	72	155	117	36	127	74–75	14.2	12.3	11.2	10.2	12.13
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J + Cu-barbital	84	80	76	79	81	155	117	36	127	74–75	13.8	12.1	...	...	...
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[a] After three recrystallizations from diluted ethanol and one from benzene-petroleum ether mixture.
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compounds were equilibrium mixtures of the N-derivatives and the O-analog since urea has also been described as being tautomeric. This possibility, however, is extremely remote. Accordingly, the reaction mixture and its products were quantitatively analyzed.

Degradative analyses were carried out on the products resulting from methods A, B, C, E, F, G, and J. The following procedure was followed and alkylamine and diethylacetic acid were obtained in all instances, establishing the products as N-substituted.

Degradative Hydrolysis of the N-Alkylbarbital Derivatives.—One-tenth mole of the appropriate N-alkylbarbital was hydrolyzed at reflux temperature with ten equivalents of hydrochloric acid. The mixture was cooled and made alkaline with sodium hydroxide (Solution I) and steam distilled into an excess of 2*N* hydrochloric acid. The solution was evaporated to dryness and the residue dissolved in 150 cc. of 1*N* sodium hydroxide. The alkaline solution was fractionally distilled with a stream of air bubbling through the mixture into a known excess of 0.1*N* hydrochloric acid and the ammonia nitrogen determined (Table II).

The higher boiling fraction was steam distilled and the distillate treated with an excess of benzenesulfonylchloride in the presence of sodium hydroxide. The benzene sulfonamide was filtered, washed with water, and recrystallized from diluted ethanol. The physical constants of the benzene sulfonamides are recorded in Table II. A portion of the benzene sulfonamide was hydrolyzed with dilute sodium hydroxide and the amine isolated and its boiling point determined (Table II).

From the first alkaline solution above (Solution I) diethylacetic acid was isolated in 88–94% yields for the different compounds. No other products were found. This compound was identified through its anilid which melted at 193°. N: calculated 7.1, found 7.0; 6.9.

Since the absence of ammonia nitrogen would be conclusive proof of N-substitution, the 1,3-dialkyl-5,5-diethylbarbituric acid derivatives were prepared through methods A, C, G, and J, and treated in the manner described above, except that the period of refluxing was doubled. The result of the hydrolysis of the tetraalkyl derivatives are reported in Table II.

The results of the degradative procedures were of particular interest since only the N-substituted product was obtained through all the alkylation procedures. The failure to obtain the O-ether by the methods employing silver must be considered in any discussion of the tautomerism of these compounds. Similar treatment of isatin resulted in the formation of the tautomeric compounds. By comparison to other imides having the $-\overset{O}{\overset{\|}{C}}-NH-\overset{O}{\overset{\|}{C}}-$ group, as phthalimide and succinimide, it would appear that the dialkylbarbiturates behave in a like manner.

Dox (5) has stated that at least one of the lactam groupings has the properties of an imide since a monosodium salt can be prepared which reacts with the alkyl halide to produce an N-alkylated derivative. After alkylation of one lactam, the resulting 1,5,5-trisubstituted barbituric acid again behaves as an imide, with the remaining lactam forming a sodium salt which reacts as before. Thus 1,3,5,5-tetraalkyl derivatives can be prepared.

In view of the above results, it was felt that a dynamic structure would satisfy more of the properties of the metal dialkylbarbiturates than does the present static graphic formula. We therefore propose the following structure for these compounds.

This structure is in agreement with the observed behavior of the metal salts of barbituric acid derivatives and allows for the maximum interpretation of tautomerism as applied to these compounds.

SUMMARY

1. Trialkylbarbituric acid derivatives have been prepared through different methods.

2. From mixed melting points and degradative analytical data, the product of the direct alkylation of barbital was shown to be N-substituted. The tautomeric O-analog could not be isolated from the reaction products.

3. A dynamic ionic structure for the metallic derivatives of the barbiturates was proposed.

TABLE II.—NITROGENOUS PRODUCTS RESULTING FROM DEGRADATIVE ANALYSIS

Compound	Ammonia N % Theoretical[a]	Ammonia N % Found	Derived Amine	Yield, %	B. p.°	N-alkylbenzenesulfonamide M. p.°	N-Analysis[b] Calcd., N, %	N-Analysis[b] Found, N, %
			Trialkylbarbituric acid Derivatives[c]					
1-Methyl		...	CH_3NH_2	...	...	29–30	8.2	7.9
1-Isopropyl		...	$(CH_3)_2CHNH_2$	93.4	33–35	25–26[d]	7.0	7.1
1-*n*-Amyl		96	$CH_3(CH_2)_4NH_2$	94.2	104	88–89	6.1	6.0
1-Benzyl		98.4	Benzylamine	97.3	185	87–88	5.6	5.7
1-Allyl		94	Allylamine	95.3	54–55	39	7.1	6.9
			Tetraalkylbarbituric Acid Derivatives[e]					
1,3-Di-*n*-amyl		0	*n*-Amylamine	97.6	104	88–89	...	...
1,3-Dibenzyl		0	Benzylamine	99.1	185	87–88	...	...
1,3-Diallyl		0	Allylamine	98.2	54–55	39	...	...

a Based on the assumption that the unsubstituted amide is converted to ammonia during the acid hydrolysis. From the alkali fusion of barbital, ammonia and potassium cyanide were isolated (2).
b Average of two determinations with a deviation less than ±0.2.
c Refers to the [illegible] derivatives.
d The phenyl [illegible] (Shriner, R. L., and Fuson, R. C., "Systematic Identification of Organic Compounds," J. Wiley & Sons, New York City, 1948, ed. 3, 179 pp.).
e Refers to the 1,3-dialkyl-5,5-diethylbarbituric acid derivatives.

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Notes

A Note on the Microscopic Characterization of the Polymorphic Forms of Ouabain*

By GEORGE L. KEENAN†,‡

IN 1929, Schwartze, Hann, and Keenan (1) published the results of an investigation of ouabain, principally for the purpose of recommending a form that could be utilized as a reliable U. S. P. Reference Standard. At that time it was suggested that ouabain, crystallized from water and containing 20% moisture, be supplanted by the drug crystallized from alcohol, to which a little ether had been added, and which contained approximately 12.5% moisture. At the present time this form of ouabain is recognized as the U. S. P. Reference Standard (2).

More recently, the writer has been interested in calling attention to the polymorphic forms of ouabain as a result of the microscopic study of the drug [illegible] aminopropyl derivatives and [illegible]ol, respectively. The results of [illegible] propanol derivatives. The comp[illegible] Benadryl and Pyribenzamine additional identity tests [illegible] replacing [illegible] specific rotation and bioassays. These [illegible]morphs are readily converted one into the other, even on an object slide. The conditions necessary for their existence and their optical crystallographic properties are presented as follows.

TESTS

Quadrilateral Plate Form.—This form, crystallized from water at room temperature, has been found to be the ouabain of commerce. The habit of the crystals is quadrilateral plates, showing very weak double refraction in parallel polarized light (crossed nicols), apparently crystallizing in the tetragonal system. The significant refractive indices of this form are: $\omega = 1.525$ and $\epsilon = 1.523$. It will be observed that these values differ only by 0.002; therefore, when the material is mounted on an object slide in either of the significant immersion liquids, the borders of the plates are almost invisible in ordinary light. By crystallizing this form from a drop of 95% alcohol on an object slide at room temperature, the rod-shaped habit of the drug is obtained (Fig. 1).

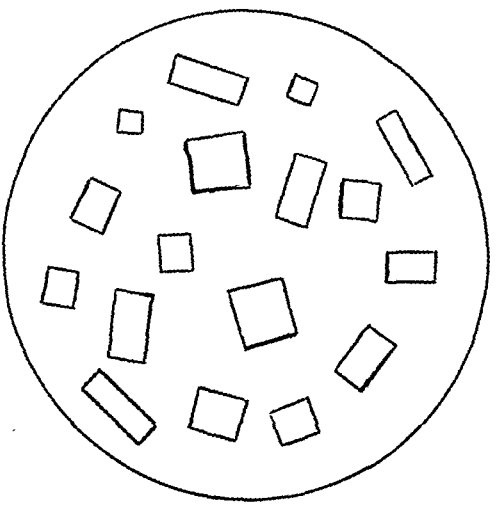

Fig. 1.—Typical habit from water.

Rod Form.—Elongated forms characterize the habit of the drug crystallized from 95% alcohol. These rods show strong double refraction, with parallel extinction and positive elongation (crossed nicols, supplemented by the selenite plate). The refractive indices are distinctly different from those

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‡ The writer acknowledges his indebtedness to the following for samples of ouabain: Committee of Revision of the Pharmacopœia of the United States of America 1940–1950; S. B. Penick & Company, New York, N. Y.; and Dr. K. K. Chen, The Lilly Research Laboratories, Indianapolis, Ind.; and to Baldwin-Wallace College, Berea, Ohio, for use of apparatus.

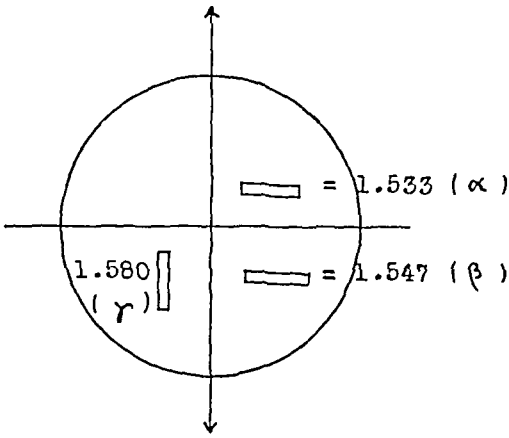

Fig. 2.—Orientation of rod-shape form (U. S. P. XIII Reference Standard).

characteristic of the quadrilateral form, and are as follows: $\alpha = 1.533$, $\beta = 1.547$, $\gamma = 1.580$ (probably orthorhombic); all $\pm$ 0.003 (Fig. 2).

This rod-shaped form is that characteristic of the U. S. P. Reference Standard, and, as already suggested, has optical crystallographic characters that are useful as microscopic identity tests.

SUMMARY

Attention has been called to the dimorphic forms of ouabain, the quadrilateral plates crystallized from water and the rods from alcohol. The optical crystallographic characters significant for rod-shaped form serve as additional identity data for the U. S. P. Reference Standard.

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A Modified Squibb Separatory Funnel for Microanalytical Work*

By CHARLES F. BICKFORD†

THE SQUIBB pear-shape type separatory funnel of about 30 cc. capacity, modified as shown in the accompanying diagram, Fig. 1, may be used advantageously in microanalytical work, when it is necessary to collect into a small volume, for critical observation and sharp separation, the usually less than a cubic centimeter of titrant solvent layer.

The modification comprises a short length of glass tubing about 2 cm. long and 0.5 cm. internal diameter, placed between the pear-shaped portion of the separatory funnel and the stopcock.

The value of the modification may be illustrated by describing a final step in the estimation of several micrograms of mercury. A standard solution of dithizone in chloroform is employed to estimate and extract simultaneously the mercury present in a small volume of approximately normal sulfuric acid. The standard solution of dithizone is adjusted so that about 2 cc. will be needed. An initial 1.4 cc. portion of the standard dithizone solution is added, from a micro-buret, to the solution in the separatory funnel. The two layers are shaken until all the dithizone has reacted with the mercury. The chloroform layer is separated. This procedure is repeated with 0.2 cc. portions of the standard dithizone solution until the presence of unreacted dithizone changes the characteristic orange-yellow color of mercury dithizonate in chloroform. This end point is reproducible to 0.1 cc., sufficiently

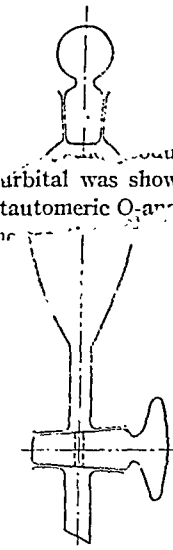

Fig. 1.—Microanalytical separatory funnel.

accurate for routine work. The chloroform layer, filling about 1 cm. length of the straight glass tube portion above the stopcock, may be easily observed. A definite color change may be ascertained rapidly.

The separatory funnel may be employed in a similar manner when dithizone is used to determine microgram quantities of other metals.

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Synthesis of Certain Derivatives of Thiols*,†

By JOHN W. BOENIGK,‡ JOHN E. CHRISTIAN, and GLENN L. JENKINS

Seven aromatic thiols have been used to prepare two series of sulfides. Thiols used were *o*-thiophenol, *o*-thiocresol, *m*-thiocresol, *p*-thiocresol, *o*-chlorobenzyl thiol, 2,4-dichlorobenzyl thiol, and 3,4-dichlorobenzyl thiol. A series was prepared of 3-diethylaminopropyl derivatives and 3-diethylamino-2-propanol derivatives. The compounds are analogous to Benadryl and Pyribenzamine type compounds with sulfur replacing the oxygen and nitrogen, respectively. The general structure of the molecule differs from these antihistamine drugs in that only one aryl group is present instead of the diaryl groups of Benadryl and Pyribenzamine. The aryl structure was varied to show the influence of substituent groups, in order that some of the thiols used would contain chlorine on the ring; others would contain methyl groups.

THE PURPOSE of the research project was to synthesize sulfur compounds analogous to Benadryl and Pyribenzamine type compounds with sulfur replacing the oxygen and nitrogen linkage, respectively. The general structure of the molecule differs from these antihistamine drugs in that only one aryl group is present instead of the diaryl groups of Benadryl and Pyribenzamine. The aryl structure was varied to show the influence of substituent groups, in order that some of the thiols used would contain chlorine on the ring; others would contain methyl groups.

It was planned to prepare two general groups of thiol derivatives, one in which the substituent was 3-diethylaminopropyl, and the other in which the substituent was 3-diethylamino-2-propanol. The diethylaminopropyl substituent has been shown to have neurotropic activity (1) and the sulfur linkage to have musculotropic activity (2), the combination of the side chain and the sulfur may have desirable antispasmodic or antihistamine activity. The purpose of the second side chain was to test the influence of such a secondary alcohol.

METHOD OF PREPARATION

The thiols used in the research project were either commercially available or were synthesized by either reduction of the corresponding disulfide or reaction of an appropriate benzyl chloride with thiourea (3). (See Tables I and II.)

* Received April 4, 1949, from the Research Laboratories, Pu[illegible] f Pharmacy, Lafayette, Ind. [illegible] Section, A. Ph. A., Jacksonville me[illegible]

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The 3-diethylaminopropyl derivatives were prepared according to the following sequence of reactions:

1. $Ar—SH + KOH \rightarrow Ar—S—K + H_2O$
2. $Ar—S—K + Cl—CH_2—CH_2—CH_2—N(C_2H_5)_2 \cdot HCl + KOH \rightarrow Ar—S—CH_2—CH_2—CH_2—N(C_2H_5)_2 + 2KCl + H_2O$
3. $Ar—S—CH_2—CH_2—CH_2—N(C_2H_5)_2 + HCl \rightarrow Ar—S—CH_2—CH_2—CH_2—N(C_2H_5)H_2 \cdot Cl$

The 3-diethylamino-2-propanol derivatives were prepared as follows:

1. $Ar—SH + NaOH \rightarrow Ar—SNa + H_2O$
2. $Ar—SNa + \underset{\diagup\!\!\diagdown O}{C}H_2—\overset{H}{C}H—\overset{H_2}{C}H_2—N—(C_2H_5)_2 + NaOH \rightarrow Ar—S—CH_2—\underset{\underset{OH}{|}}{CH}—CH_2—N(C_2H_5)_2 + NaOH$

The compounds listed in Table III were all prepared by the same method. The thiol (0.1 mole) was dissolved in 50 cc methyl alcohol and a solution of KOH 14.4 Gm. (0.2 mole plus 10% excess) in 50 cc. methyl alcohol added. This mixture was refluxed one-half hour. The 1-diethylamino-3-chloropropane HCl (18.6 Gm.) (0.1 mole) was dissolved in 25 cc. methyl alcohol and added slowly to the refluxing mixture. The refluxing was continued for four hours, the solution cooled and filtered through a Büchner funnel. The methyl alcohol was removed by distillation at atmospheric pressure and the residual oil washed with water and dissolved in benzene. The benzene solution was dried with anhydrous Na_2SO_4, the benzene removed by vacuum distillation, and the residual oil distilled under reduced pressure.

The 1-diethylamino-3-chloropropane was prepared by two methods (8, 9).

Method A.—Diethylamine 117 Gm. (1.6 mole) was added slowly to trimethylene chlorobromide 126 Gm. (0.8 mole) while the reaction mixture was maintained at 30°. The mixture was allowed to react at 35–40° for three hours, and stand overnight. The salt formed (diethylamine HBr) was removed by washing with 80 cc. cold water. The residual oil was added to 500 cc. cold 2 *N* HCl and the unreacted trimethylene chlorobromide removed with ether. The acid solution was made alkaline with K_2CO_3,

TABLE I.—DISULFIDES PREPARED

	M. P., ° C.		% Yield	
	Obtd.[a]	Lit.[b]	Obtd.	Lit.
2,2′-Dichloro-dibenzyl disulfide[c]	88–89	89–90 (4)	75	96
2,2′,4,4′-Tetrachloro-dibenzyl disulfide[c]	71–72	74–75 (4)	70	84
3,3′,4,4′-Tetrachloro-dibenzyl disulfide[c]	91–92	94–95 (4)	72	89

[a] Uncorrected.
[b] Corrected.
[c] The disulfides were prepared by the procedure described by Christian, *et al.* (4):
1 2,2′-Dichloro-dibenzyl disulfide: o-Chlorobenzyl chloride (30 Gm.) was dissolved in 150 cc. refluxing ethyl alcohol. To the hot alcoholic solution a warm alcoholic solution of Na_2S_2, prepared by dissolving 21.8 Gm. of $Na_2S \cdot 9H_2O$ and 2.9 Gm. of sulfur in 210 cc. of hot ethyl alcohol, was added slowly. The refluxing was continued for 4 hr., the mixture filtered through a Büchner funnel and the filtrate allowed to cool. The precipitate was recrystallized from ethyl alcohol.
2. 2,2′,4,4 and 3,3′,4,4′-tetrachloro-dibenzyl disulfide were prepared by the same procedure using chloride (30 Gm.), $Na_2S \cdot 9H_2O$ (17.9 Gm.) and sulfur (2.4 Gm.).

TABLE II.—THIOLS PREPARED

	Method Used	Yield, %	B. P., ° C./Mm.	Refractive Index, ° C.	3,5-Dinitrobenzoate Esters M. P., ° C.[a]
o-Chlorobenzyl thiol[b]	A	Quant.	120–121/25	1.5650/28	112.5–113.5
	B	73	100–104/13	1.5658/28	112.5–113.5
2,4-Dichlorobenzyl thiol[b]	A	Quant.	151–152/29	1.5993/29	110.5–111.5
	B	68	133–135/16	1.5992/29	110.5–111.5
3,4-Dichlorobenzyl thiol[b]	A	Quant.	170–171/31	1.6000/29	97.0– 98.0
	B	78	139–141/13	1.6012/29	97.0– 98.0

[a] Uncorrected.
[b] The thiols were prepared either by (A) reduction of corresponding disulfides using zinc and hydrochloric acid, or (B) reaction of appropriate benzyl chloride with thiourea (3). Both methods were used for all three thiols.
Method A—Reduction of Disulfide.—A modification of the procedure by Allen, *et al.* (5) was used The appropriate disulfide (50–60 Gm.) was dissolved in 200 cc. of glacial acetic acid. The solution was heated to boiling and 30 Gm. of zinc dust and 90 cc. of l in divided portions so as to maintain a rapid evolution of hydrogen at all times. was removed and diluted with water. If the diluted sample formed a clear solution ion was discontinued, and the warm solution filtered through a Büchner funnel. olution, additional amounts of zinc and hydrochloric acid were added until it did so.
The filtrate was diluted with twice its volume of distilled water, and the thiol layer which separated, removed by means of a separatory funnel and the aqueous portion extracted with ether. The ether was evaporated on a water bath and the residue combined with the crude thiol and dri The dried thiol was distilled under reduced pressure.
Method B—Thiourea Method.—A *et al.* (3) was used. A mixture of o-chlorobenzyl chloride 79.3 Gm. (0.5 mole), thiour hol 250 cc. was refluxed on a steam bath for 12 hr. A solution of NaOH, 30 Gm. (0.75 mole) in 300 cc. of water was added, and the mixture refluxed an additional 4 hr. The thiol layer was separated and the aqueous layer was acidified with dilute sulfuric acid (7 cc. conc. H_2SO_4 to 50 cc. water), and extracted with 75 cc. benzene. The benzene extract and the crude thiol were combined and washed with water, then dried with benzene was removed by evaporation, and the thiol distilled under reduced pressure.
the thiols were prepared by a method given by Wertheim (12). In a test tube were placed enzoyl chloride, (0.015 mole) of the thiol, and 4 drops of pyridine. The test tube was heated until fumes of HCl ceased to appear (about 10 min.). In certain cases it was necessary to heat the test tube in a steam bath for an additional 10 min. A few drops of water were added to the mixture, then an excess of pyridine, and the mass stirred vigorously until it solidified. The reaction mixture was then filtered and dried on a porous plate. The material was recrystallized from dilute ethyl alcohol.

TABLE III.—DIETHYLAMINOPROPYL SULFIDE HYDROCHLORIDES

Thiol Used	B. P., ° C./Mm. Amine	Yield Amine, Gm.[a]	M. P., ° C. Amine HCl[b]	Nitrogen[b] Calcd.	Nitrogen[b] Found	Cl[c] as HCl Calcd.	Cl[c] as HCl Found
o-Chlorobenzyl thiol	190–193/12	20 (80%)	83–84	4.54	4.46	11.49	11.45
2,4-Dichlorobenzyl thiol	165–168/4	22.5 (73%)	116–117	4.08	4.02	10.31	10.29
3,4-Dichlorobenzyl thiol	169–172/3	24.9 (81%)	75–76	4.08	4.03	10.31	10.24
Thiophenol	141–143/6	18.1 (81%)	61–63	6.26	5.90[d]	13.67	13.15
o-Thiocresol	150–153/5	16.9 (71%)	126–127	5.11	4.93	12.95	12.98
m-Thiocresol	168–173/13	20.8 (88%)	65–66	5.90	5.41[d]	12.95	12.52
p-Thiocresol	155–158/7	19.4 (81%)	97–98	5.11	5.08	12.95	12.90

[a] Recrystallizing solvent: ethyl acetate
[b] Nitrogen determined by a semimicro Kjeldahl method (7).
[c] Chlorine determined by the method described by Blicke and Zienty (6).
[d] Amine hydrochlorides were very hygroscopic, N analysis made on free base.

the oil separated, and the aqueous layer extracted with ether. The combined ether extracts and oil were dried with anhydrous K_2CO_3. After evaporation of the ether, the residual oil was distilled under reduced pressure. The yield obtained was 59 Gm. (49% theoretical), the boiling point was 68–70° at 20 mm.

Method B.—A solution of γ-diethylaminopropanol 78.5 Gm. (0.6 mole) in 130 cc. of chloroform was added slowly with cooling to a solution of thionyl chloride 145 Gm. (1.21 mole) in 600 cc. of chloroform. The mixture was refluxed for three hours, after which the solvent and excess thionyl chloride were removed by distillation. The residue was treated with 150 cc. of a 40% solution of NaOH and extracted with 1000 cc. of ether. The ether solution was dried over anhydrous Na_2SO_4 and the ether evaporated. The residue distilled as a colorless oil 62–64° at 19 mm., yield 79.3 Gm. (88% theoretical). Gilman and Shirley (9) reported a yield of 73% theoretical, boiling point 73–75° at 20 mm.

1-Diethylamino-2,3-epoxypropane was also prepared by two methods (10, 11).

Method A.—A mixture of epichlorohydrin (46.3 Gm.), diethylamine (30.0 Gm.) and water (1.0 Gm.) was well mixed while maintaining the temperature at 28–30° for four hours. The resultant oil was shaken with 50 cc. 20% K_2CO_3, then with 60 cc. of a 35% NaOH solution for one hour. The oil was then shaken with 50 cc. of a 50% KOH solution. The resultant oil after separation from the KOH solution was distilled under reduced pressure. The yield obtained was 35.8 Gm. (55.4% theoretical). The boiling point of the product was 40–50° at 8 mm.

Method B.—A mixture of epichlorohydrin (111.2 Gm.), diethylamine (86.4 Gm.), and 3.6 Gm. water was stirred vigorously for six hours maintaining the temperature of the reaction mixture at 28–30°. The reaction mixture was then cooled to 20° and a solution of NaOH (50 Gm. in 91 cc. H_2O) was added slowly, meanwhile maintaining vigorous stirring. The reaction mixture was stirred for an additional forty minutes and poured into 200 cc. water. The top layer was separated and the aqueous layer extracted with ether. The ether extracts plus the oil were dried over KOH pellets. The ether layer was separated from the KOH and the ether removed by evaporation. The residual oil distilled at 53–56° at 17 mm., the yield being 75 Gm. (50% of theoretical). Gilman reported a 62% yield and the boiling point as 62–65° at 20 mm.

The compounds listed in Table IV were prepared as follows: The sodium hydroxide 8.8 Gm. (0.2 mole plus 10% excess) was dissolved in 50 cc. of water and the thiol (0.2 mole) added to the NaOH solution. The 1-diethylamino-2,3-epoxypropane 25.8 Gm. (0.2 mole) was dissolved in 50 cc. of water, and added slowly with vigorous stirring to the thiol-NaOH solution. The mixture was stirred for an additional four hours, and the resultant oily product after being washed with water, was dissolved in 50 cc. of benzene. The benzene solution was dried with anhydrous sodium sulfate and the benzene removed by evaporation under reduced pressure. The residual oil was distilled under reduced pressure. All attempts to convert these amines to crystalline hydrochlorides failed.

SUMMARY

1. Seven diethylaminopropyl and seven diethylaminohydroxypropyl derivatives of thiols have been prepared and characterized.

TABLE IV.—1-DIETHYLAMINO-2-HYDROXY-PROPYLSULFIDES

Thiol Used	B. P., ° C./Mm.	Yield Amine, Gm.	Nitrogen[a] Calcd.	Nitrogen[a] Found	Refractive Index (/° C.) Amine
o-Chlorobenzyl thiol	190–195/4	17 (60%)[b]	4.85	4.56	1.5435/30
2,4-Dichlorobenzyl thiol	210–215/4	42.8 (66%)	4.34	4.23	1.5522/27
3,4-Dichlorobenzyl thiol	210–215/4	44.3 (69%)	4.34	4.14	1.5515/30
Thiophenol	168–171/4	41.3 (86%)	5.85	5.79	1.5412/25
o-Thiocresol	154–157/1	35.6 (65%)	5.50	5.32	1.5408/30
m-Thiocresol	168–172/3	32.0 (60%)	5.50	5.37	1.5376/30
p-Thiocresol	168–172/3	35.0 (64%)	5.50	5.28	1.5371/30

[a] Nitrogen determined by semimicro Kjeldahl method (7).
[b] Made using 0.1 molar quantities, others made using 0.2 molar quantities.

2. Sufficient quantities of these compounds have been prepared for pharmacological study.

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Assay of Heparin. III. Measurement of the End Point by Physical Means. Influence of Surface on Clotting*,†

By R. H. K. FOSTER

In the assay of heparin using beef or sheep plasma the degree of clotting has been estimated by visual inspection. This estimate, visual density, has been correlated with light transmission as measured photoelectrically and with the weights of dried clots. Good agreement was obtained. At times the surface of thoroughly cleaned and stored Pyrex tubes interfered with the action of heparin in some unknown manner so that very irregular end-point responses were obtained. Concentration-response clotting curves obtained using freshly cleaned Pyrex glass tubes were similar to those obtained using silicone-coated glass tubes or cellulose nitrate tubes.

IN THE ASSAY method previously described (1) the end point was considered to be that amount of heparin which would just permit the formation of a 50 per cent clot. Since a 50 per cent clot is seldom obtained in any tube, the concentration of heparin corresponding to a 50 per cent clot must be calculated by interpolation from a clotting curve. The degree of clotting in the various tubes has always been estimated visually. It is the purpose of this paper to correlate visually estimated degrees of opacity with opacity measured photoelectrically and with the weights of dried clots. In the course of this work some very marked irregularities were observed when clotting took place in Pyrex glass tubes but were completely eliminated when clotting was allowed to proceed in silicone-coated Pyrex tubes or in cellulose nitrate tubes. A preliminary report of this investigation has been made elsewhere (2).

MATERIALS, CRITERIA, AND PROCEDURES

Opacity determined by inspection is termed visual density. This, representing the degree of coagulation, was estimated in terms of an arbitrary scale, 0 to 4+, with several intermediate values (1).

The transmission of light, here measured photoelectrically, is a reciprocal function of opacity, the latter reflecting the degree of coagulation. Results are reported in terms of per cent transmission of light since this simplified the plotting of curves. However, for convenience, the term optical density is employed. Since optical density is $-\log I/I_0$ the light transmission scale has been inverted to make these curves parallel to those for visual density and clot weight. The instrument accounts for light absorbed (or scattered) by the plasma as well as by the clot itself in contrast to the eye which discounts the plasma color when inspecting for visual density.

In reporting the weights of dried clots it is not presumed that the weights represent pure fibrin. High-speed centrifuging was employed to compact the fibrin clots but it is more than probable that the clots still contained some plasma and/or serum which when dried would add to the weight of the dried fibrin. Certain characteristics of the clot such as size and number of fibrin fibrils, their specific gravity, and their "malleability" must influence the ease of packing during centrifugalization and hence entrained material. It is probable that different concentrations of heparin alter these factors to such an extent that the proportionate amount of packing varies and the ratio of the amount of fibrin present to the entrained impurities is not strictly constant. Nevertheless, it is assumed that the clot weights as determined represent reasonable parallelism to the "true" weight of the fibrin.

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† The heparin used in this investigation was kindly supplied by Hoffmann-LaRoche, Inc., Nutley, N. J.

Plasma.—Beef and sheep plasmas were employed. The blood when collected was rendered incoagulable with 4 mg. sodium citrate per ml. of blood. After centrifuging for thirty minutes at 2000 r. p. m., the supernatant plasma was siphoned off and frozen until used.

Heparin.—Heparin No. 1 previously reported (3) was dissolved in 0.9% saline in a concentration of 10 γ per ml. for tests on beef plasma and 20 γ per ml. for tests on sheep plasma.

Calcium Chloride.—A concentration of 10 mg. per ml. was employed.

Preparation of Tubes.—Tubes were filled as in the regular assay procedure, that is, in the order: heparin + plasma + saline + calcium. The total volume was either 2.0 ml. or 3.0 ml. The larger volume was required for the electrophotometer used and the proportion of all ingredients was maintained in the same ratio. The plasma volume was 1.0 or 1.5 ml. and the calcium chloride solution 0.2 or 0.3 ml. The tubes were 13 by 100 mm. Unless otherwise stated clotting was allowed to proceed for one hour at room temperature.

Colorimeter Readings.—A Fisher electrophotometer was used with the initial null adjusted to 100% with distilled water as the standard. Most plasmas, beef or sheep, after dilution 1:1, gave readings in the range of 95 ± 2% light transmission. A red filter was used with the rheostat set at *A*.

Clot Weights.—The clots were broken and chopped up in the tubes using a dental spatula. The entire contents were transferred to 13 × 100-mm. cellulose nitrate tubes (CNT), washed several times with distilled water, and placed in a Sorvall Super-Speed centrifuge. The machine was run for one hour at full speed (about 12,000 r. p. m.). The fragments of the clot became compressed into a rubber-like deposit and were removed after three or four rinsings in water. The compressed clot was pressed out firmly on a dry filter paper, placed on a small aluminum weighing boat and dried overnight over calcium chloride. Weighings were made on a Roller-Smith torsion balance. In most tests one hour was allowed for clotting and one hour for centrifuging. The manipulative procedures unfortunately consumed as much as half an hour or more, depending on the number of tubes employed (usually 8 or 16), so that some tubes stood much longer than one hour during the clotting phase.

Besides the above procedure, the clotting reaction was also carried out in the cellulose nitrate tubes during centrifugation. When this was done, the centrifuge was started as quickly as possible (a few seconds) after mixing the ingredients. The tubes were centrifuged one hour.

RESULTS

Section 1. Correlation of Visual Density, Optical Density, and Clot Weight

All three of these measurements were made on each tube. Curves for each of the measurements obtained from a series of tubes containing graded concentrations of heparin have been plotted in Figs. 1 to 4. For graphing, the ordinate scales were adjusted so the three different curves would be approximately superimposed.

Sheep Plasma.—Figure 1 shows the results of allowing the clotting of sheep plasma to proceed in cellulose nitrate tubes for one hour before centrifuging. The concentration of heparin ranged from 0 γ to 22 γ. With no heparin the plasma was considered fully clotted and on the curve for visual density this is represented by 4+. However, by inspection it could be seen that the clot was *less dense* than in the tube containing 16 γ of heparin. The change in optical density in this range is indicated by the curve for light transmission. This effect of heparin has already been discussed (4). As one would expect, the two curves descend substantially together. The curve for optical density does not reach the base line since the unclotted plasma absorbs some

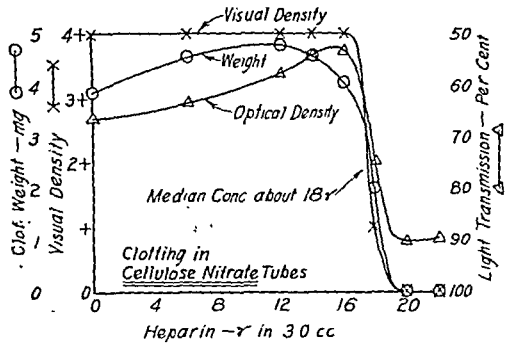

Fig. 1.—Sheep Plasma—SKL (Expt. 79).

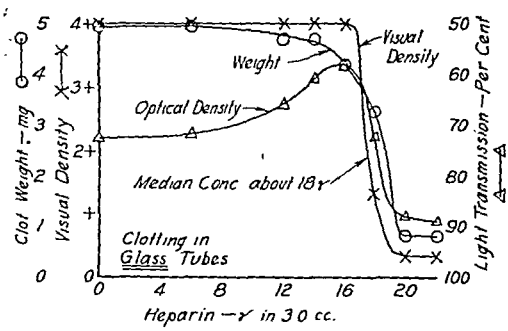

Fig. 2.—Sheep Plasma—SKL (Expt. 79).

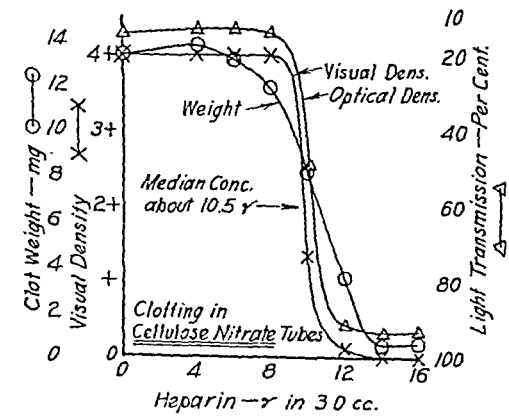

Fig. 3.—Beef Plasma—BKO (Expt. 80).

light.[1] The weight of the clots shows a progressive increase paralleling for some distance the curve of optical density, but the weight begins to decrease *before* the peak in optical density is reached. This break in the weight curve before the peak of the optical density curve was not seen in all experiments. It may reflect an error although the weight curve is smooth.

In Experiment 82 on sheep plasma SIJ (graph not shown), the clot-weight curve remained practically horizontal and descended *with* the curves for visual and optical density (measured in the cellulose nitrate tubes). That is, there was no premature "break" in the weight curve as referred to above. Otherwise, the curves were identical with those of Fig. 1.

Figure 2 shows the results of tests carried out in Pyrex tubes in the same manner as those in cellulose nitrate tubes shown in Fig. 1. Note that the initial tube (no heparin) has somewhat lower optical density (increased transmission). This is explained in Footnote 1. The forms of the curves are the same as in Fig. 1 except that the weight curve does not rise initially. It "breaks" before the peak of the optical density curve as in most other experiments. There was some residual clot present as shown by the failure of all three curves to reach the base line. The median concentration (i.e., CC_{50}) was about 18 γ (or 12 γ per ml. of plasma). In Experiment 82, the results with sheep plasma SIJ in Pyrex tubes were substantially the same except that the weight curve showed a very slight descent up to the "break" which was synchronized with the curves for optical and visual densities; and also, the curves approached the base line more closely. The median dose for both curves was 16 γ. The descending segments of the curves in Experiment 82 were practically identical in position.

Beef Plasma.—Tests with beef plasma in cellulose nitrate and Pyrex tubes are shown in Figs. 3 and 4. In general the curves have the same characteristics as those for sheep plasma. The curve for optical density shows very little rise during the first part. The light was only 10% to 15% whereas with sheep plasma this stage had a light transmission of around 60% or 70% where comparable changes would be proportionally greater. If beef plasma were diluted several times it seems probable that the sensitivity would have increased and the curves approached those for the sheep plasma. The slight increase in the weight of the clot with 4 γ of heparin (Fig. 3) can probably be attributed to technical error. The weight curves "break" before the peak of the optical density (and visual density) just as with sheep plasma. In the Pyrex tubes (Fig. 4) 14 γ and 16 γ fail to produce complete inhibition and with the latter concentration there is an actual increase in clot density as shown by all three curves. Though slight this is probably real since this phenomenon has been repeatedly obtained and is demonstrated in greater detail in the succeeding experiments. The median concentration in the Pyrex tubes is 1.0 to 1.5 γ higher than in the cellulose nitrate tubes. Much greater differences have been found from time to time and will be illustrated later.

[1] The colorimeter readings as per cent light transmission are lower with the cellulose nitrate tubes than with Pyrex tubes because their internal diameter is about 2 mm. greater.

In Experiment 85 (not illustrated) in which a different beef plasma, BHI, was used, the results differed slightly. Part of this differing was due to the lower optical density of the final clot. The minimum light transmission in glass tubes was 21% compared to only 10% in Experiment 80 and the curve rose in a manner somewhat similar to the curves for sheep plasma shown in Figs. 1 and 2. The weight curve showed a slight descent before the "break" occurred, the latter being simultaneous with the curves for visual and optical density. In the descending portion the three curves did not agree as well as those in Fig. 4. The median dose was about 13.5 γ. Tests in cellulose nitrate tubes were not run. In Experiment 84 (Fig. 6) the curves for

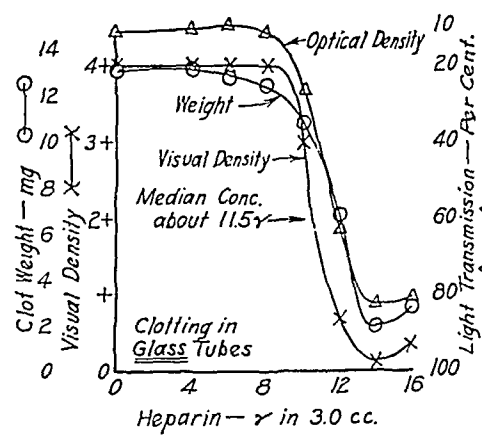

Fig. 4.—Beef Plasma—BKO (Expt. 80).

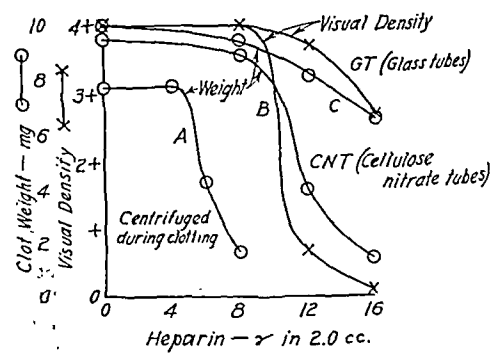

Fig. 5.—Beef Plasma—BKO (Expt. 72).

Pyrex tubes showed poor correlation in the dosage range covered.

Centrifugation during Clot Formation.—When plasma is centrifuged at high speed during the process of clotting, the clot weights obtained are materially less. This is illustrated in Fig. 5 and in Table I. In the graph, comparison is made with clot weights obtained from plasma allowed to clot for an hour before centrifuging. The difference in the initial height of curves *A* and *B* is attributed mainly to greater compaction of the clot which is thrown down by centrifugal force as fast as formed. That is, there is less

TABLE I.—WEIGHTS OF DRIED CLOTS FROM UNHEPARINIZED PLASMA

Expt. No.	Plasma Sample	Cellulose nitrate tubes Centrifugation During Clotting	Cellulose nitrate tubes Centrifugation After Clotting	Glass tubes Centrifugation After Clotting
72	BKO	7.74	9.45	10.03
73	BKO	8.09	10.10	9.28
				9.95
74	BKO	8.11	...	10.37
		8.08		10.23
				9.75
				10.23
75	BKO	7.70	...	9.77
		7.86	...	8.96
76	BKO	7.87	9.55	9.74
		7.89	8.95	9.51
			9.94	10.29
			10.01 (S)[b]	
80	BKO	7.97	8.89	8.72
	Average:	7.92	9.55	9.76
84	BHI	...	...	6.29
85	BHI	...	...	6.56
77	SKL	2.76	3.15 (S)[b]	3.09
79	SKL	3.35[a]	2.59	3.30
82	SIJ	...	5.70	5.58

[a] Three-minute delay in starting centrifuge.
[b] (S) Silicone-coated tubes.

Experimental Conditions:

Weights: in mg./ml. of original plasma.
BKO, BHI: Beef plasma: SKL, SIJ: Sheep plasma.
Plasma age: varied from 3 minutes to $2^1/_4$ hours after thawing.
Clotting time: 1 or 2 hours' duration.
Centrifuging time: 1 hour except in Expt. 72 which was $^1/_2$ hour.
Clotting temperature: room temperature except for 3 tubes in Expts. 73 and 74 clotted at 37°.
Plasma volume: 1.0 ml. or 1.5 ml. (total volumes 2.0 or 3.0 ml.). Data calculated to 1.0 ml.
The variations in these conditions caused no apparent differences in the clot weights.

entrained plasma or serum present. Table I illustrates this in the clotting of heparin-free plasma. Under the conditions of the test (i.e., Fig. 5.) the heparin was much more effective, the median dose (CC_{50}) being only about half that obtained in the cellulose nitrate tubes where clotting occurred before centrifugation. The time difference could partly explain this since it has been shown (4) that as plasma ages less heparin is required. However the magnitude of the change is not explained on the basis of any data nor is any explanation forthcoming at the moment.

The results shown in Table I, which have already been referred to, were gathered together from a number of experiments in which heparin was used and in which observations on visual density and optical density were made. When tubes were allowed to clot during centrifugation the latter two measurements naturally could not be made. Clotting was allowed to take place during centrifugation to see if there would be any greater compaction than when the centrifuging was done later. This evidently occurs since for plasma BKO the average reduction in clot weight is 17%. This 17% presumably represents the weight of dried entrained serum. But it is probable that the observed weight of the clot is still in excess of the true weight of the fibrin present by more than this 17%.

Section 2. Irregularities Attributable to Surface Effect

Ever since heparin assays were started in 1940 there have been occasional instances where an irregular end point was obtained; that is, clotting (partial or complete) was noted in tubes containing more than the presumed CC_{50} of heparin. These were generally ascribed to technical errors. The end points otherwise have generally been fairly sharp and in line with the curve shown in (1) and with a "spread" much less than indicated by Kuizenga for beef plasma. Recently, however, several series of tests showed very unusual distribution of the erratic end point. Figures 5 to 8 clearly demonstrate the irregularities and will be described in detail.

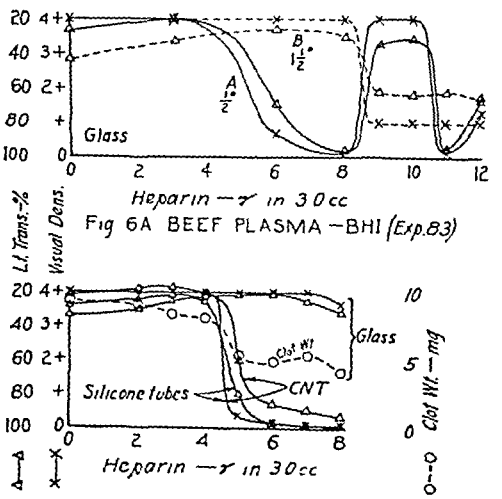

Fig. 6.—Beef Plasma—BHI (Expt. 84).

The effect of the glass surface is shown in Fig. 5 (*C*). Although the plasma was the same as used for the experiments in Figs. 3 and 4, the result, that is, the CC_{50} (by interpolation), was quite different. The inhibiting action of heparin was much less. The estimated CC_{50} is about 18 γ (12 γ/ml. of plasma). This is much higher than the 11.5 γ (7.7 γ/ml. of plasma) found in Fig. 4. The CC_{50}'s obtained in cellulose nitrate tubes were the same in both experiments.

In Fig. 6 the results obtained in silicone-coated and cellulose nitrate tubes show fairly good agreement for both optical and visual densities. The CC_{50} estimated from each curve ranged from 4.5 to 5.0 γ (or 3.0 to 3.3 γ/ml. of plasma). Clot weights were not determined. In glass tubes there was almost complete clotting up through at least 8 γ concentration. The clots were weighed but did not correlate well with the optical and visual densities; actually the weight curve more nearly resembles the optical and visual density curves for clotting in silicone-coated and cellulose nitrate tubes.

Figure 6 (*A*) shows results obtained using only glass tubes. The two curves marked *A* are for measurements of visual and optical density with plasma aged one-half hour after thawing. Since the dosage intervals were wide up to 8 γ the curves as drawn between 3 γ and 8 γ are probably not correct, but should be steeper. At 8 γ the clotting was almost nil but with concentrations of 9 γ and 10 γ there was complete clotting. At 11 γ the curves again approached the base line and at 12 γ rose to nearly a 50% clot. The exact history of these glass tubes prior to use was not recorded except that they were lipless Pyrex tubes and all received the usual acid-dichromate cleaning treatment (1) and were stored for a few days. The clotting was observed closely during the experiment and the relative order of clotting density was noted for the different concentrations of heparin at nine, thirteen, eighteen, and thirty minutes. To a considerable extent this order can be correlated with the curves of Fig. 6 (*A*). The results, omitting the thirteen- and eighteen-minute observations, were:

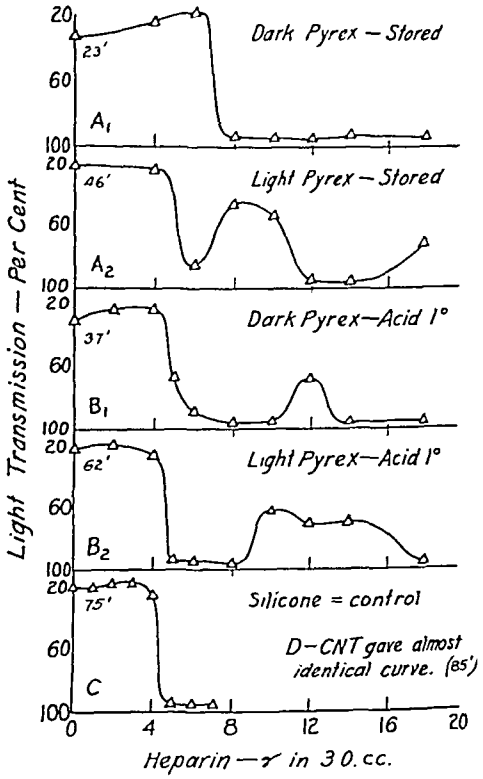

Fig. 7.—Beef Plasma—BHI (Expt. 87).

for the unrecalcified plasma. However, by visual inspection a trace of clot could be seen in the tubes containing 8 γ, 12 γ, and 14 γ of heparin. This series would seem valid except for the comparison with silicone and cellulose nitrate tubes (*C* and *D*) where the median dose was found to be 4.5 γ. The fresh acid treatment for one hour did not alter the response as shown in curves B_1 and B_2.

A few tests were made to determine the influence of alkali and acid on tubes. Light tinted Pyrex

	Decreasing density ⟶							
At nine minutes:	0	10	9	12	11	3	6	8
At thirty minutes:	0	3	10	9	12	6	8	11
Final, two hours:	0	3	10	9	12	6	11	8 (from curves)
Expected order:	0	3	6	8	9	10	11	12

The tests were repeated with the plasma aged one and one-half hours, again with abnormal although different results (Fig. 6 (*A*), curves *B*).

An attempt was made to determine what influenced the greater irregularity. It was noted that lipped Pyrex tubes had a dark or a light tint when examined closely with side lighting. At first it was thought that the darker tubes were chiefly responsible for the irregularities. Accordingly a series of tests were made with the light and dark tubes separately. The results are shown in Fig. 7. Stored tubes were first employed and then a few were treated with the cleaning solution and used. In these particular tests the stored dark tubes gave the most uniform results as shown in curve A_1. The median concentration of heparin in this test was about 7 γ. The curve leveled off at about the value

tubes were employed. The results are shown in Fig. 8. Silicone tubes served as the control but the amount of plasma available was small so only three concentrations of heparin were employed. In *A*, a dotted line indicates how the curve could be expected to run. In *B*, stored tubes were used. The length of storage was not recorded but it was more than a week. The tubes were kept in dust-free containers. In *C* and *D*, the results with three and one-half hours' soaking in cleaning solution are given. In *C*, the tubes were rinsed in the customary manner with tap and distilled water and finally with redistilled water and then dried in an oven. The tubes used for *D* were similarly treated except that before the final rinsing they were allowed to stand overnight in distilled water. The thought was that possibly the chromium ion (5) might be playing a role and

that overnight soaking would remove the last traces. In both cases the curves are comparable to the silicone curve. This would seem to indicate that tubes should be freshly cleaned with cleaning solution, at least not longer than twenty-four to forty-eight hours before the tests are to be made. If this is the case, one hour is not sufficient soaking time as shown in Fig. 7. In *E* and *F* are shown the effects of rinsing the acid-cleaned tubes with 10% sodium hydroxide. Soaking overnight in water after the alkali treatment did not improve the results. Presumably some alkali adhered to the glass permanently.

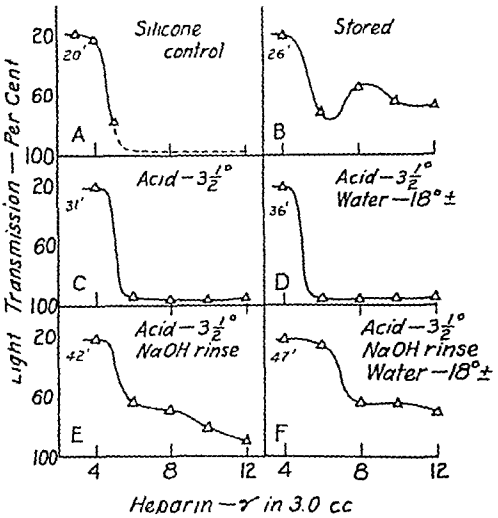

Fig. 8.—Beef Plasma—BHI (Expt. 88).

A few other tests were made on the effect of acid or alkali. In Experiment 85 a few Pyrex tubes freshly cleaned in the acid-dichromate cleaning solution were used. With beef plasma BHI concentrations of heparin of 8 γ, 10 γ, 12 γ, and 15 γ gave just traces of clot in all tubes. In stored tubes with the same doses, the clots were 4+, 4+, 3+ and +(−), respectively. In Experiment 86, plasma BHI was used. Five minutes' soaking in acid cleaning solution resulted in the same erratic response as in stored tubes.

In Experiment 89, using plasma BKO, stored tubes gave erratic responses while results using silicone tubes were normal (cf. Fig. 7, A_2 and *C*). Tubes soaked twenty-four hours in cleaning solution gave curves similar to the silicone series. Again stored tubes gave erratic responses with very flattened descending curves for visual and optical density.

Experiment 90 was conducted using sheep plasma SN. The erratic behavior was considerably less than that noted for beef plasma. In stored tubes a slight film of clot adhered to the walls of two tubes whereas with the same heparin concentrations in freshly cleaned tubes (forty-eight hours in acid; rinsed the day used) there was no film with these same concentrations. One series (Experiment 90-D) was run with narrower concentration intervals using stored tubes. The curves for both optical and visual density were flatter than usually obtained for sheep plasma. The heparin concentration spread for the descending portion of the two curves was about 60%, that is, the first tube with no clot had 60% higher heparin concentration than the last tube showing complete clotting.

In still other cases in experiments where regular assays were being conducted, sheep plasma showed a tendency to form a film of clot on the tube walls with concentrations of heparin well beyond the end point and to a degree approaching that seen using beef plasma (3). However, in the many sheep plasmas tested (and usually using "stored" tubes) this result has been the exception rather than the rule.

In the light of present data it may be concluded that if Pyrex tubes are soaked in strong, sulfuric acid-dichromate cleaning solution overnight, and within twenty-four to forty-eight hours of use, the results are more likely to resemble those obtained in silicone or cellulose nitrate tubes when either beef or sheep plasma is employed.

CONCLUSIONS

1. In most tests there was close correlation between visual density, optical density, and clot weight in the descending segments of the curves. This range includes the criterion for the end point, the 50 per cent clotting concentration (CC_{50}). Validity of the end point obtained by visual inspection in heparin assays is thus established.

2. Serious irregularities have been encountered in the clotting of recalcified heparinized beef plasma and to a much lesser extent in sheep plasma when clotting took place in Pyrex tubes that had been stored for some time. The use of silicone-coated tubes or cellulose nitrate tubes completely eliminated the irregularity.

3. The present observations imply that Pyrex glass tubes should be freshly cleaned with sulfuric acid-dichromate cleaning solution within a day or so of their use and with at least several hours' soaking before thorough rinsing with water.

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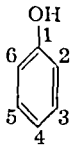
Comparative Toxicity of Certain *t*-Butyl Substituted Cresols and Xylenols*

By W. A. McOMIE, HAMILTON H. ANDERSON, and F. M. ESTESS

Of the compounds studied, 2-*t*-butyl 4-methylphenol, 2-*t*-butyl 5-methylphenol, 2,4-di-*t*-butyl 5-methylphenol, 2,6-di-*t*-butyl-4-methylphenol and 3,5-dimethylphenol have a lower degree of acute toxicity than phenol itself. However, 2,4-dimethyl-6-*t*-butylphenol is unique in its toxicity to the rabbit, being as lethal by simple application to the intact skin as by the oral route of administration. This is apparently a species susceptibility since rats and guinea pigs are less susceptible to the compound by both the oral and the skin route. It is concluded that the dialkyl phenols are more irritant locally than the trialkyl phenols studied.

THE COMPOUNDS of chief interest[1] in this study are important as oxidation inhibitors. They are all derivatives of phenol and for sake of brevity will be designated by the position of the alkyl groups on the ring relative to the phenolic hydroxyl group according to the following scheme.



2-*t*-Butyl 4-methylphenol (2B4M)
2-*t*-Butyl 5-methylphenol (2B5M)
2,4-Di-*t*-butyl 5-methylphenol (24B5M)
2,6-Di-*t*-butyl 4-methylphenol (26B4M)
6-*t*-Butyl 2,4-dimethylphenol (24M6B)
3,5-Dimethylphenol (35M)

Parallel experiments were conducted in certain instances with phenol, carvacrol, and thymol.

All of the compounds contained less than 1 per cent of impurities. Carvacrol, 2B5M and 24M-6B were liquids at 25°. The others were solids. The physical properties of several compounds were reviewed by Pardee and Weinrich (1).

The purpose of this study was to determine the relative local and systemic toxicities of the compounds and to estimate their relative hazard to man.

EXPERIMENTAL

Intragastric Administration to Mice.—Cottonseed oil solutions (1–4%) of the phenols were administered to previously fasted (sixteen to twenty-four hours) stock white, male, mice (weighing from 15 to 25 Gm. each) by means of a blunt-tipped 18-gauge needle attached to a syringe. Volumes given varied from 0.2–0.8 cc. (Table I).

After intragastric administration of cottonseed oil solutions of all compounds other than phenol, the mice reacted in essentially the same manner. There usually was depression to the point of prostration at the higher dose levels. At the lower dose levels no effect other than depression was observed. This was the general observation after administration of 2B4M, 2B5M, 24B5M, 24M6B, 26B4M, 35M, and thymol. Phenol in doses as low as 280 mg./Kg. caused all mice to exhibit muscular tremors and generalized body twitchings. Deichmann and Witherup (3) described these tremors or twitchings in rats, rabbits, and cats after phenol administration. These authors also found that *o*-, *m*-, and *p*-cresols cause these twitchings but were less severe with the cresols than with phenol itself. From our results it would appear that further substitution in the cresol molecule causes this effect to disappear.

The pathologic effects produced in mice by these compounds consisted of irritant action on the gastroenteric tract as evidenced by hemorrhages in the region of the small intestine. Severe edema and congestion of the lungs were usually found grossly after both acute and delayed deaths. There was no microscopic evidence of myocardial damage. Delayed deaths occurred after exposures to some of these compounds. An observation period of ten days was chosen arbitrarily. Some animals died as long as ten days after administration of the compounds. Phenol, 35M, and thymol were exceptions to this delayed-death effect. In no instance after application of these three compounds did death occur after forty-eight hours. The tri-substituted compounds, however, were particularly noteworthy in this delayed death effect. In the case of 24M6B and 26B4M over 50% of the deaths occurred ninety-six hours after application of the compounds. Livingston (4) found that delayed deaths (five days and more after administration) took place with carvacrol and thymol after intragastric administration to rabbits.

Administration to the Skin of Rabbits.—The liquid compounds (2B5M, 24M6B, and carvacrol) were placed in rubber cuffs and the solid compounds on gauze held in place by a flexible wire screen according to the method of Draize, *et al.* (5). In the case of the liquid compounds the exposures were for six hours. The severe local effects of 2B5M and carvacrol were evident after this time and 24M6B was lethal. All of the solids were allowed to remain on the skin for twenty-four hours (Table II). The compounds were also applied in ether by flowing the ether solution on to the shaved back of the rabbit (Table III).

From the above results the following generalizations were made concerning these compounds after administration topically to rabbits:

* Received September 20, 1948, from the Division of [illegible] and Experimental Therapeutics, University of [illegible] San Francisco 22, Calif.
[illegible] Development Co., Emeryville, [illegible] port of this study.

TABLE I.—ACUTE INTRAGASTRIC TOXICITY OF ALKYL PHENOLS IN COTTONSEED OIL SOLUTION TO MICE

Dose, Mg./Kg.	Mortality Ratio (10 Days' Observation)							
	2B4M	2B5M	24B5M	24M6B	26B4M	35M	Thymol	Phenol
280	0/10	...	...	3/10	...	0/10	...	0/5
420	1/10	...	...	4/5	1/10	1/10	...	0/5
620	7/10	2/5	1/10	5/10	4/10	4/10	0/5	4/5
940	5/10	1/10	2/10	4/5	4/10	9/10	0/5	5/5
1400	...	6/10	3/10	...	6/10	...	3/10	...
2100	...	5/5	9/10	...	...	...	3/5	...
LD_{50}[a]	700	1080	1420	530	1040	620	1800	540[b]
Standard[a] Error LD_{50}	±35	±93	±108	±36	±204	±52	±224	...

[a] Graphic method of Miller and Tainter (2) was used to obtain the approximate LD_{50}'s and their standard errors.
[b] Estimate.

TABLE II.—CUFF EXPOSURES OF RABBITS TO ALKYL PHENOLS

Compound	Dose[a]	Duration of Exposure, Hr.	Effects Noted
Carvacrol	2.7	6	Immediate irritation, becoming erythematous. Leather-like appearance within 4 hr. Complete necrosis and eventual sloughing and scar formation. Died 72 hr. after exposure.
2B4M	2.2	24	Similar local effects as with carvacrol. Died 72 hr. after exposure.
2B5M	2.9	6	Similar local effects as with carvacrol. No apparent systemic effect other than that due to severe burn of the skin. Survived.
2B5M	3.9	6	Similar picture as above. Survived.
35M	2.6	24	Similar effect as with 2B5M. Survived.
24B5M	4.5	24	Only slight irritation of skin (mild erythema without subsequent necrosis). No apparent systemic effect.
26B4M	3.3	24	No evidence of skin irritation. No apparent systemic effects.
24M6B	2.9	6	Died within 6 hr. from beginning of exposure. No local erythema or edema. Lung grossly edematous and congested.
24M6B	3.9	6	Same effect as above. Both animals had sufficient lung edema to have caused death.

[a] Dose is given in cc./Kg. for the liquid compounds (2B5M, carvacrol, and 24M6B) and in Gm./Kg. for the solid compounds. One rabbit was used at each dose level.

TABLE III.—APPLICATION OF ETHER SOLUTIONS OF ALKYL PHENOLS TO THE SKIN OF THE RABBIT

Compound	Dose, Gm./Kg.	Effects Noted
Phenol[a]	0.42	Skin turned parchment-like within 24 hr. No apparent systemic effect. Complete necrosis of superficial layers after 10 days. Survived.
Thymol	0.42	Similar picture to that of phenol.
35M	0.42	Similar picture to that of phenol.
2B4M	0.42	Similar picture to that of phenol.
2B5M[b]	0.42	Similar picture to that of phenol.
24B5M	0.42	Some erythema and superficial sloughing but no severe tanning effect as seen with phenol.
26B4M	0.42	Similar picture to that of 24B5M.
24M6B	0.08	Died 36 hr. after application. No local effect. Edematous lungs found upon gross autopsy.

[a] Liquefied phenol, U. S. P. XIII, was used.
[b] Given undiluted.

1. 2B4M, 2B5M, 35M, and carvacrol are severely irritant and resemble phenol in their coagulative effect on the skin. Carvacrol and 2B4M may cause fatalities through exposure of relatively large areas of the skin. This may be due to secondary effects from the severe chemical burn rather than from systemic absorption of the compounds themselves.

2. Although 24M6B is only mildly irritant it is readily absorbed from the skin and causes death through systemic action, rather than indirectly from skin injury.

3. The two solid tri-substituted alkylated phenols (24B5M and 26B4M) are the least irritant, and evidently little is absorbed from the solid form.

4. None of the other compounds had any apparent systemic effect after topical applications of a dose 5 times greater than the lethal dose of 24M6B (see Table V).

Effect of Alkyl Phenols on the Eyes of Rabbits.—Table IV summarizes the results of exposure to the eyes of rabbits. In each case 0.03 cc. of the solution was applied directly to the cornea.

The irritation to the rabbit's eye of these compounds places them in similar categories as shown previously for their action on the skin. Thymol, 35M, 2B4M, and 2B5M are all extremely irritant; 24B5M and 24M6B are less irritant but still dangerously irritant to mucous surfaces. 26B4M, due to

its lack of solubility, could not be given in a water-miscible solvent. Its lack of irritation in diallyl phthalate (6) might indicate that it had a much greater affinity for the relatively bland medium in which it was dissolved rather than for the corneal or conjunctival membrane. Allowing for this inability to demonstrate irritation in the case of 26B4M, it is apparent that all the compounds are injurious to the eye but are not particularly more so than thymol.

guinea pigs and rats by simple application without rubbing. The lethal doses obtained by this method are shown in Table V.

In rabbits there was a remarkable paralleling of toxicity by the oral and skin routes of administration. However, the rat and the guinea pig are not only less susceptible than the rabbit to intragastric administration of the compound but also show less likelihood of toxic effects through skin absorption.

TABLE IV.—APPLICATION OF 0.03 CC. ALKYL PHENOLS TO THE RABBIT CORNEA

Compound	Concentration	Result[a] in 1 Hr.	24 Hr.	72 Hr.	Fluorescein[b] Test, 24 Hr.
2B4M	40% in ethylene glycol	31	80[c]	80[c]	[d]
2B5M	Undiluted	32	78	80[c]	100
24B5M	40% in ethylene glycol	13	9	2	90
24M6B	Undiluted	0	43	53	90
26B4M	40% in diallyl phthalate	0	0	0	0
35M	40% in ethylene glycol	31	80[c]	80[c]	90
Thymol (control)	40% in ethylene glycol	32	80[c]	80[c]	100

[a] Score of Draize, *et al.* (5), used (1944); the larger the number, the greater the injury to the mucous membranes.
[b] Numbers under fluorescein refer to percentage staining of corneal surface with fluorescein and indicate extent of damage to corneal epithelium.
[c] After 24 hr. the eye was closed so completely that injury could not be evaluated by the scoring method. The maximum score possible (110) was approached in the case of 2B4M, 2B5M, 35M, and Thymol. Permanent clouding of cornea resulted in case of 24M6B.
[d] Eye was completely closed and could not be opened to instill the fluorescein.

TABLE V.—COMPARATIVE LETHAL DOSES OF 24M6B TO RABBITS, RATS, AND GUINEA PIGS BY THE ORAL AND SKIN ROUTES OF ADMINISTRATION

	Oral			Skin		
	Rabbits	Rats	Guinea Pigs	Rabbits	Rats	Guinea Pigs
Lethal dose,[a] cc./Kg.	0.055	1.4	0.42	0.055	10.7[b]	7.1

[a] Lethal dose as defined by Deichmann and LeBlanc (7) as first lethal dose in the series.
[b] No deaths occurred. This was highest amount given.

Comparative Toxicity of 24M6B by Skin and Oral Routes of Administration.—The results of skin application to rabbits indicated that 24M6B had a systemic toxicity considerably above any of the other compounds when applied by this route. In addition it was not as irritant as some of the other compounds possibly rendering its hazard even greater since lack of irritation would not provide warning and could lead the person exposed to a false sense of security. Accordingly, a study of the comparative toxicity to rabbits, rats, and guinea pigs by the oral and skin routes of administration was made. The method of Deichmann and LeBlanc (7) was used for determining the approximate lethal doses by these routes. This method consists briefly of giving a single animal a dose differing by 50% from the preceding dose given to another animal. The approximate lethal dose by their definition is the lowest dose of the series that kills. These authors showed that for a group of compounds this approximate lethal dose varied from the LD_{50} by about ±30%. Employing this method (with the exception that in some cases more animals were employed at each dose in the lethal range), the following results were obtained. Compounds were given orally either undiluted or dissolved in propylene glycol. Topical applications were made to the previously clipped area of the backs of guinea pigs and rabbits and to the abdominal surface of rats. The undiluted compound or ether solutions of each (when small amounts were applied) were given to

The rat, in particular, is quite resistant by both the oral and the skin routes.

The signs of toxicity in rabbits were, first, depression, then dyspnea accompanied by râles and frothing at the nostrils. Convulsions ensued terminally probably due to asphyxia as the result of profound lung hemorrhage and edema, easily seen grossly at autopsy.

Signs of poisoning in rats and guinea pigs were essentially the same as in rabbits. The lung changes were not as severe as in rabbits even with lethal doses. The stomach wall and intestines were highly inflamed in both guinea pigs and rabbits after oral administration of the undiluted compound or as a 10% solution in propylene glycol. The evidence on species difference in susceptibility to 24M6B seems unmistakable. An indication that other compounds were not as toxic as 24M6B to the rabbit was seen when single rabbits survived intragastric doses of 0.62 Gm./Kg. of 26B4M and 0.94 Gm./Kg. of 2B5M, respectively, without any apparent effect. Both of these amounts are more than 10 times the intragastric LD_{50} for rabbits of 24M6B.

DISCUSSION

Aside from the previous communication on the toxicity of 24M6B by Treon and Cyama (8) no other data concerning the toxicity of these compounds have appeared in the literature. Related alkyl

phenols have been investigated by Lamson, *et al.* (9, 10) and studies have been made comparing the toxicity of thymol and carvacrol by Livingston (4) and Caujolle and Franck (11). Deichmann and Witherup (3) have compared the toxicity of phenol and the *o*, *m*-, and *p*-cresols.

The substituted cresols and xylenols studied, with the exception of 24M6B, in the rabbit, are more comparable in toxicity to thymol than to phenol itself. It would seem, in view of the widespread use of thymol in pharmaceutical products, that these compounds should present no particular hazard if used with caution.

The unique toxicity of 24M6B in the rabbit is of interest because its lethal dose by the skin and oral routes is nearly identical. The mechanism of this toxicity and the reason for its ease of penetration through the intact rabbit skin are as yet unexplained. This compound should be treated with utmost caution in view of its extreme toxic effects in the rabbit.

SUMMARY

1. The range of acute intragastric LD_{50} for mice of cottonseed oil solutions of certain *t*-butyl substituted compounds was from 530–1800 mg./Kg. The relative order of toxicity by this route from most toxic to least toxic was 6-*t*-butyl 2,4-dimethylphenol, phenol, 3,5-dimethylphenol, 2 *t*-butyl 4-methylphenol, 2,6-di-*t*-butyl 4-methylphenol, 2-*t*-butyl 5-methylphenol, 2,4-di-*t*-butyl 5-methylphenol, and thymol. The compounds may be considered as of intermediate toxicity between phenol and thymol in so far as the intragastric route for mice is concerned.

2. Carvacrol, thymol, phenol, 2-*t*-butyl 4-methylphenol, 2-*t*-butyl 5-methylphenol, and 3,5-dimethylphenol were severely irritant to the skin of rabbits upon prolonged exposure. The trisubstituted compounds (2,6-di-*t*-butyl 4-methylphenol, 2,4-di-*t*-butyl 5-methylphenol, and 6-*t*-butyl-2,4-dimethylphenol) while irritant are not in the same category as the dialkyl derivatives or phenol itself.

3. The compound 6-*t*-butyl 2,4-dimethylphenol, though only mildly irritant to the skin of rabbits, caused death in this species with as small a dose as 52 mg./Kg. applied to the skin. The LD_{50} of this compound to rabbits is approximately the same by either the oral or the skin routes. Rats and guinea pigs, however, were less susceptible to its toxic action after intragastric administration and much less susceptible after percutaneous application.

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Microchemical Identification of Amidone*

By ROBERT C. WATSON† and MAX I. BOWMAN‡, §

Amidone may be identified microchemically through formation of crystals with certain reagents. Tests with palladium chloride, potassium ferrocyanide, and bromine water are described and a table of the crystal-forming action of these reagents on the salts of 34 alkaloids and synthetic drugs is presented.

SINCE the discovery of amidone (6-dimethylamino-4,4-diphenyl-3-heptanone)[1] in the 1930's and its subsequent introduction into medicine as an analgesic, considerable interest has been shown toward it by the field of clinical medicine (1).

Because of its addiction liability and morphine-like action, amidone falls within the purview of the U. S. Bureau of Narcotics as a restricted narcotic drug. It is, therefore, desirable to have a relatively simple and rapid method of identifying samples which are seized from illicit channels.

Amidone may be readily identified microchemically. Its reaction with 197 reagents, including many of the common so-called "alkaloidal re-

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§ The writers wish to acknowledge their appreciation to Raymond A. Bevins, Identification Section, Alcohol Tax Unit, Louisville, Ky., for assistance in preparing the photomicrographic plates.
[1] Also known in the drug field as dolophine or methadon.

agents" was examined. Of these, 43 gave crystals, the remainder giving only amorphous precipitates or none at all. Only 3 reagents were selected as giving well-defined crystals of characteristic shapes suitable for identification purposes, the others being rejected on the basis of atypical crystals, or because of similar crystal formation with many common alkaloids.

TABLE I.—REACTIONS OF AMIDONE GIVING CRYSTALLINE PRECIPITATES[a]

Reagent	Type of Crystals
Ammonium acetate	Rectangular plates (R)
Ammonium carbonate	Bundles and rosettes of plates
Ammonium hydroxide	Dense masses of rectangular plates
Ammonium iodide	Dense rosettes of feather-like crystals
Ammoniacal nickel acetate (2)	Clumps of indistinct crystals
Ammonium oxalate	Needles and rods (R)
Ammonium sulfide	Rectangular plates (R)
Ammoniacal silver nitrate (2)	Masses of poorly defined crystals
Barium acetate	Rectangular plates (R)
Barium chloride	Mostly small plates (R)
Barium hydroxide (sat. soln.)	Fine root-like branching crystals (R)
Bromine water (sat. soln.)	Flat plates with notched ends, later rosettes of plates
Cadmium iodide	Bundles of small, branching, rod-like crystals
Calcium chloride	Rectangular plates (R)
Hydrobromic acid (conc.)	Mostly diamond-shaped plates and tablets
Hydriodic acid (47%)	Bundles and rosettes of plates
Iodine water (sat. soln.)	Small "X"-shaped plates
Iodine-Potassium iodide	Rectangular and notched plates
Mercuric chloride	Bundles of rod-like plates
Mercuric nitrate (sat. soln.)	Bundles of flat rods
Magnesia mixture (2)	A few rectangular plates and tablets (R)
Nickel acetate	Some rectangular plates (R)
Palladium chloride (in dil. HCl)	Twinned spearhead-like crystals, later fern-like branches
Potassium acetate	Tablets and plates (R)
Potassium-Bismuth iodide	A few rectangular plates (R)
Potassium bitartrate	Rods and tablets (R)
Potassium chromate	Irregular rosettes of plates
Potassium ferrocyanide	Rods and rosettes of rod-like crystals
Potassium iodide	Dense rosettes of fern-like crystals
Potassium nitrite	Masses of overlapping plates
Potassium oxalate	Mostly rectangular plates and fragments (R)
Saccharin	Overlapping plate formations
Silver iodide-Potassium iodide (1:15)	A few rosettes of irregular plates
Sodium acetate	Irregular plates
Sodium bicarbonate	Irregular chain formation of plates
Sodium borate	Network of indistinct crystals
Sodium carbonate	Clumps of indistinct crystals
Sodium fluoride	Mostly hexagonal tablets (R)
Sodium iodide	Dense rosettes of branching needles
Sodium phosphate (dibasic)	Rectangular plates (R)
Sodium silicate (sat. soln.)	A few small square plates (R)
Sodium sulfite	Irregular plates
Sodium tungstate	Masses of indistinct crystals

[a] Crystal types marked (R) appear, on preliminary examination, to be reagent crystals thrown from solution.

EXPERIMENTAL

The tests were made in the usual manner by placing the reagent drop on a microscopic slide adjacent to the sample drop and allowing the two drops to flow together. The slide was allowed to stand for a short time and then was examined under a microscope (magnification ×100) for crystal formation.

Photomicrographs were made of characteristic crystals using a 4 x 5-in. Speed Graphic plate camera. The lens was removed and the microscope draw-tube extended into the camera bellows, the image being brought to sharp focus on the ground glass screen by rotating the micrometer head of the microscope.

Aqueous amidone hydrochloride solutions of 1:50, 1:200, 1:500, and 1:1000 concentrations were tested. In no instances were the slides scratched to hasten crystal formation. The use of cover glasses was avoided since they tended to cause the formation of distorted crystals.

PREPARATION OF REAGENTS

Palladium Chloride.—(Approximately 1% in dilute HCl.) Make up 1 Gm. $PdCl_2 \cdot 2H_2O$ to 100 ml. with dilute HCl (35 ml. HCl, *s. g.* 1.19, *q. s.* 100 ml. with distilled water); warm gently until solution is complete.

Potassium Ferrocyanide.—(Approximately 5% aqueous solution.) Make up 5 Gm. $K_4Fe(CN)_6$

$3H_2O$ to 100 ml. with distilled water (3). This reagent should be made up fresh each day since it slowly decomposes on standing.

Bromine Water.—(Dilute solution.) Make up 3.5 ml. saturated bromine water to 100 ml. with distilled water. This reagent should be made up frequently.

Amidone reacted with approximately 1% aqueous solutions of 43 reagents to give crystalline precipitates with the results shown in Table I.

Only amorphous precipitates were obtained with ammonium molybdate, antimony trichloride, arsenic chloride, barium nitrate, bismuth nitrate, cadmium chloride, calcium hydroxide, chromic acid, chromium nitrate, cobalt nitrate, cobalt thiocyanate (blue ppt.), cupric nitrate, 2,4-dinitro-phenylhydrazine, ferric nitrate, Froehde's Reagent (3) (white ppt.), gold chloride, iridium chloride, Kraut's Reagent (3), Lanthanum nitrate, lithium carbonate, lithium chloride, lithium nitrate, Marme's Reagent

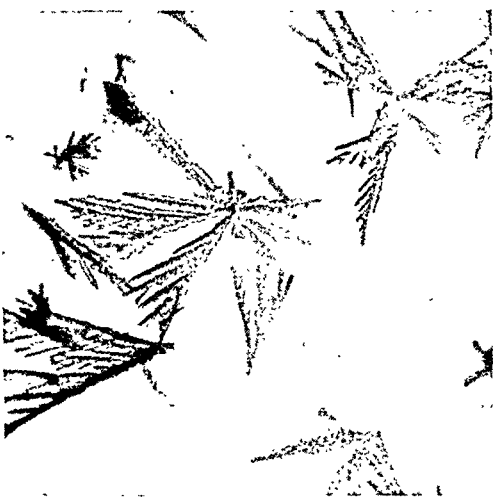

Fig. 1.—Amidone 1:50—palladium chloride crystals.

(3) (grayish white ppt.), Mayer's Reagent (3) (white ppt.), Millon's Reagent (3) (white ppt.), molybdic acid, nickel nitrate, picric acid, picrolonic acid, Phenylhydrazine-HCl, phosphomolybdic acid, phospho-tungstic acid, platinum chloride, potassium acid phthalate, potassium carbonate, potassium chloride, potassium cyanide, potassium dichromate, potassium ferrocyanide, potassium hydroxide, potassium permanganate, potassium phosphate, potassium sulfide, potassium-zinc-iodide, rhodium chloride, ruthenium chloride, silico-tungstic acid, silver acetate, silver sulfate, sodium-benzene-sulfonate, sodium bichromate, sodium-cobalt-nitrite, sodium nitro-prusside, sodium peroxide, sodium phospho-molybdate, sodium sulfide, sodium thiocyanate, stannous chloride, tetra-chloro-phthalic acid, Wagner's Reagent (3), zinc-chloro-iodide.

Crystals Best Suited for Identification.—In making the tests one drop each of a $^1/_{50}$, $^1/_{200}$, $^1/_{500}$, and $^1/_{1000}$ aqueous solution of amidone hydrochloride was placed on a microscopic slide together with an equal drop of the reagent. The slide was allowed to stand for a short time until crystal formation occurred and then microscopic examination was made under a magnification of 100×.

Palladium Chloride.—These crystals form almost at once from an amorphous precipitate in the $^1/_{50}$ solution (Fig. 1), and somewhat slower in the $^1/_{200}$ solution (Fig. 2). In greater dilutions the crystals were very slow in forming and resembled double-headed flint spearheads (Fig. 3). These crystals

Fig. 2.—Amidone 1:200—palladium chloride crystals.

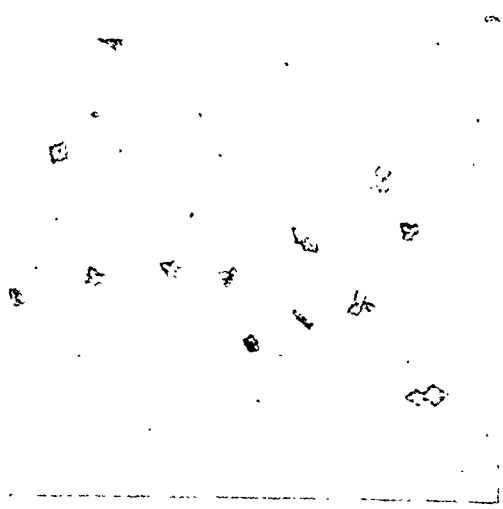

Fig. 3.—Amidone 1:500—palladium chloride crystals.

show brilliantly under polarized light. They grow to considerable size, forming rosettes of brown fern-like plates or fragments. One notable feature is the fact that the rod-like crystal growth occurs on one side only of each arm and at an angle of approximately 30° from the main axis. This was found to be the most satisfactory crystalline test.

Potassium Ferrocyanide.—Crystals form at once from a clear solution in the $^1/_{50}$ concentration (Fig. 4). At first these crystals appear as bundles of needle-like rods curving outward at the ends, having somewhat the appearance of sheaves of grain. Later rosettes of plates were formed. Crystals were not formed in the more dilute solutions. The three dimensional growth of these crystals may best be observed under polarized light.

Bromine Water.—Many small crystals formed at once in all solutions, but those in the $^1/_{200}$, $^1/_{500}$, and $^1/_{1000}$ solutions were best for identification purposes (Fig. 5). This test was improved by adding a drop of dilute HCl to the amidone solution and stirring before adding the bromine water. The crystals at first appear to be single plates having notched ends in such a manner as to resemble the letter "N," when slightly out of focus. Later they develop into rosettes of rod-like plates.

Fig. 4.—Amidone 1:50—potassium ferrocyanide crystals.

Fig. 5.—Amidone 1:200—bromine water crystals.

Crystal Formation with Other Drugs.—The selected reagents were tested for crystal formation with 34 alkaloidal salts and synthetic drugs. Eight crystalline and 13 amorphous precipitates were obtained as indicated by Table II. These crystals have distinctive shapes not easily confused with those formed with amidone.

CONCLUSIONS

Amidone may positively be identified by microscopic examination of crystals formed with palladium chloride, potassium ferrocyanide and bromine water. These reagents give relatively few crystalline precipitates with other common alkaloids and synthetic drugs.

The search for crystal-forming reagents outlined by the authors was not an exhaustive one. It seems likely, therefore, that characteristic crystals suitable for identification purposes might be formed with other reagents.

TABLE II.—MICROCHEMICAL REACTIONS OF 34 ALKALOIDS AND SYNTHETIC DRUGS WITH 3 REAGENTS

Drug Tested	Reagents: Palladium Chloride	Potassium Ferrocyanide	Bromine Water
Dicodid	...	...	...
Dilaudid	...	...	...
Demerol	...	...	...
Cocaine	..[a]	..[b]	...
Morphine	...	A	...
Narcotine	A	...	A
Codeine	...	...	A
Narceine	..[a]	C[a]	...
Thebaine	A	...	...
Papaverine	A	A	A
Dionin	...	...	...
Pantopon	...	...	...
Heroin	...	...	...
Atropine	...	...	...
Caffeine	C	...	...
Brucine	C	...	...
Strychnine	C	C	...
Saccharin	...	...	...
Stovaine	...	C	A
Quinine	...	A	A
Novocaine	...	...	..[c]
Alypin	...	...	A
Phenacetin	...	...	...
Sulfon-Methane	...	...	...
Na-Phenobarbital	C	...	...
Nembutal	C	...	...
Seconal	A	...	...
Ephedrine-Amytal	...	...	...
Digitalin	...	...	...
Nitroglycerin	...	...	...
Scopolamine	...	...	...
Strophanthin	...	...	...
Ergotrate	...	...	...
Nicotine	...	...	...

(A—amorphous precipitates; C—crystalline precipitates)

a Disagreement with the findings of Stephenson (4) previously reported. The concentrations used above in most instances differed from those used by Stephenson.

b Rosenthal (5) obtained six-sided crystals with cocaine using ferrocyanide as a reagent.

c Saturated bromine water gives bundles of crystals with novocaine.

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Spectrophotometric Assay Method for Pyranisamine Maleate*

By LUISE T. ANDERSON, W. C. GAKENHEIMER, CHARLES ROSENBLUM, and E. H. SMITH

A spectrophotometric assay method for pyranisamine maleate, the nonproprietary name of a new antihistaminic compound being marketed under the trade-mark "Neo-Antergan" maleate, in various pharmaceutical preparations is described together with a discussion of the ultraviolet absorption characteristics of this compound. Tabulated results indicate that assay deviations from the expected composition are in most cases less than 5 per cent. The constancy of the absorption spectra of pyranisamine maleate in these preparations indicates the stability of the antihistaminic in these preparations.

PYRANISAMINE maleate is the nonproprietary name of a new antihistaminic compound being marketed under the trade-mark "Neo-Antergan" maleate. The incorporation of this compound into pharmaceutical preparations such as tablets, ointments, elixirs, and parenteral solutions is being investigated.

The salt is obtained by neutralizing one mole of the free base N-(α-pyridyl)-N-(*p*-methoxybenzyl)-N′,N′-dimethylethylenediamine with one mole of maleic acid. Being an aromatic substituted derivative of ethylenediamine, pyranisamine exhibits an ultraviolet absorption spectrum sufficiently intense and characteristic to distinguish it from substances commonly present in pharmaceutical preparations, a fact which serves to simplify the assay of such preparations. This was ascertained by studying the absorption spectral characteristics of the maleate in aqueous solutions over a wide *p*H range, in 20 per cent ethyl alcohol and in a mixture of absolute ethyl alcohol and petroleum ether. The latter solvents were included in the investigation because they facilitate the dissolution of certain types of pharmaceutical preparations. The contribution of maleic acid to the absorption spectrum of the salt was also studied in aqueous solutions. The spectrophotometric study will be described below, together with illustrative applications to the assay of typical pharmaceutical preparations.

* Received March 3, 1949, from the Research Laboratories, Merck & Co., Inc., Rahway, N. J.

SPECTROPHOTOMETRIC STUDY

Materials.—Four samples of pyranisamine maleate were used to determine its absorption spectrum. Melting points and carbon, hydrogen, and nitrogen data are listed in Table I. Monsanto maleic anhydride was used in the preparation of maleic acid solutions. The ethyl alcohol and petroleum ether (b. p. 50–60°) employed as solvents in certain measurements were of reagent grade, as were the materials required for the preparation of certain of the buffers (*p*H's 4.4, 7, and 11). Other buffers were of the "Parstains" phosphate type commercially available.

TABLE I.—PROPERTIES OF PYRANISAMINE MALEATE SAMPLES

Sample	M. P., ° C.	C	H	N
A	101.5–103	63.03	6.78	10.46
B	101	62.93	6.42	10.69
C	101	62.85	7.08	10.74
D	101–102	62.41	6.65	10.76
Theoretical	...	62.83	6.78	10.87

Measurements.—Absorption spectra (optical density vs. wave length) of solutions were measured in 1-cm. quartz cells with a Beckmann, Model DU. Quartz Spectrophotometer, the solvents themselves being used as reference liquids. Solutions were prepared for observation by dissolving 2 mg. of the maleate (accurately weighed on a microbalance) in 100 ml. of solvent, which was usually distilled water or an aqueous buffer, though 20% ethyl alcohol and a 50-50 mixture of absolute ethyl alcohol and petroleum ether were also investigated. Spectra in aqueous solutions were determined at *p*H's ≃4 ($10^{-4}N$ HCl), 4.4 (acetate buffer), 5 ("Parstains" phosphate buffer), 5.5 ("Parstains" buffer), 6.0

("Parstains" buffer), 6.5 ("Parstains" buffer), 7 (monobasic phosphate-alkali buffer) and 11 (tetraborate-alkali buffer). All *p*H's were measured with a Cambridge Research Model *p*H Meter after solution of the pyranisamine maleate. The spectral characteristics of maleic acid itself were determined by dissolving ≃2 mg. of anhydride (microbalance) in 100 ml. of water, dilute acid, or alkali to yield unbuffered solutions of *p*H 3.5, 5.3, 7.1, 9.5, and 11.2.

Ultraviolet Absorption Spectra.—The spectrum of pyranisamine maleate changes with acidity and with solvent. In aqueous solution at *p*H <5.5, it consists of two bands located at 2400–2430 Å. and 3075–3100 Å., whereas above 6, a third band is present at 2215–2240 Å., probably due to a shift to longer wave lengths of an acid band existing below 2100 Å. No attempt was made to follow the transition between *p*H's 5.5 and 6. The spectra in distilled water and in 20% alcohol were practically identical with the *p*H 5.5 curve; but in alcohol-petroleum ether solution, three band maxima were found at 2475, 2850, and 3075 Å. The intensities of the several absorption bands are summarized in Table II which lists their wave-length locations in Angström units (Å.) and extinction coefficients ($E^{1\%}_{1\,cm.}$).

this method permits a direct analysis without the necessity of performing customary chemical separations of the compound sought. For example, if the absorption spectrum of a substance is known, and a characteristic intense band chosen, its presence and concentration in a given mixture can be determined merely by measuring the optical density, at this wave length, of an appropriately diluted solution of the preparation against a suitable reference liquid. If the other ingredients of the mixture do not themselves absorb in this region, the reference solution may be the liquid solvent itself. On the other hand, if other components do absorb appreciably, the reference liquid must be constituted identically with the diluted sample, with the exception of the substance being determined. This must be done in order to correct for the light absorption and scattering (turbidity) by the other ingredients of a preparation. In only one type of preparation studied was the direct assay vitiated by the extreme absorption of an inert component; and extraction of this material (an essential oil) with ether was necessary before the assay could be completed.

The absorption band most suitable for the determination of pyranisamine maleate in aqueous medium is located at ≃2435 Å. It is quite intense and, except for a slight shift to shorter wave lengths

TABLE II.—ABSORPTION BANDS OF PYRANISAMINE MALEATE IN SEVERAL MEDIA

Medium	Max. Å.	$E^{1\%}_{1\,cm.}$	Max. Å.	$E^{1\%}_{1\,cm.}$	Max. Å.	$E^{1\%}_{1\,cm.}$
*p*H 4.0	...	...	2400	427	3100	156
*p*H 4.4	...	...	2415	425	3080	145
*p*H 5.0	...	...	2430	423	3070	130
*p*H 5.5	...	...	2430	425	3075	128
*p*H 6.0	2215	465	2435	415	3065	126
*p*H 6.5	2230	424	2440	419	3065	121
*p*H 7	2240	448	2435	436	3055	129
*p*H 11	2230	426	2485	450	3140	124
Distilled water (unbuffered; *p*H ≃ 5.5)	...	...	2435	419	3065	125
20% Alcohol	...	...	2435	423	3075	131
Petroleum ether–absolute alcohol[a]	2475	477	2850	86	3075	122

[a] The spectrum below 2250 Å. was not explored due to the opacity of the solvent employed.

Typical complete spectra are plotted in Figs. 1 (*p*H's 4 and 5.5), 2 (*p*H's 6 and 11), and 3 (20% alcohol and alcohol-petroleum ether), which show optical density vs. wave length. The first two figures also show the absorption spectra of maleic acid in equimolecular concentrations. The spectrum of the free acid is rather insensitive to changes in acidity. It consists of simple end absorption, which decreases gradually as the *p*H increases up to ≃7, beyond which it remains essentially constant. It is clear that the discrete spectrum of the salt, and changes therein, reflect the properties of the pyranisamine itself rather than those of the maleic acid.

APPLICATIONS

Principle.—The absorption spectrophotometric evaluation of pharmaceutical preparations for pyranisamine maleate content, which will be described below, is a typical application of ultraviolet spectrophotometry to the analysis of chemotherapeutic (1) and other types (2) of compounds. In many systems,

at lower *p*H's, the extinction coefficient remains quite constant in the *p*H range 4–6.5. Furthermore, the same spectra are found in 20% alcohol and in distilled water (*p*H ≃5.5), indicating an insensitivity of the spectrum to the presence of alcohol and salts from the buffer mixtures. Accordingly, for all work in water or 20% alcohol, an over-all average $E^{1\%}_{1cm.}$ of 422 was employed in calculating concentrations of pyranisamine maleate. For solutions in 1:1 absolute alcohol–petroleum ether, the 2475 Å. band with an $E^{1\%}_{1cm.}$ of 477 was employed. In actual practice, the use of buffers was dispensed with since solutions in distilled water automatically fell in the *p*H range 5–6 The *p*H of aqueous solutions was always checked, however, to assure a *p*H <6.5.

Preparations.—The spectrophotometric assay method for pyranisamine maleate was tested with the pharmaceutical preparations described below. Materials used in compounding these samples were all U. S. P. grade or equivalent.

Tablets.—Two strengths of coated tablets were prepared which contained 25 and 50 mg. of the male-

ate per tablet, respectively, in addition to the customary ingredients such as sugar, starch, certain salts, and a trace of dye. Placebo tablets of identical composition, except for the omission of pyranisamine maleate, were also prepared for reference purposes.

Elixirs.—Several 0.5% elixirs were compounded with alcohol, essential oils, and aromatic elixir. Blank elixirs of identical composition, except for the pyranisamine salt, were always prepared to correct for the incomplete transparency of other components. It was found necessary to use the same lots of oil and aromatic elixir for the blank as were used in formulating the pyranisamine elixir itself because of the varying absorption spectral patterns of different lots.

Anhydrous Petrolatum Ointments.—Several ointments containing 5% of pyranisamine maleate in white petrolatum were prepared to test the applicability of the spectrophotometric assay method to this type of formulation. No blank ointment was required since the pyranisamine maleate was extracted from the ointment base.

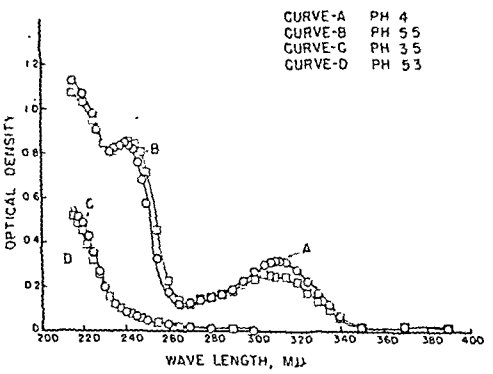

Fig. 1.—Absorption spectra of Pyranisamine Maleate (curves *A* and *B*; 2 mg./100 ml.) and Maleic Anhydride (curves *C* and *D*; 0.488 mg./100 ml.).

Anhydrous Water-Soluble Ointments.—Several 5% ointments of this type were prepared. The base consisted of Carbowax and propylene glycol, and is essentially the same as the Ointment No. 11 base described by Macek, *et al.* (3).

Aqueous Emulsion-Type Ointments.—Five per cent emulsion-type ointments were formulated with wax, higher alcohols, emulsifying agent, glycerin, and water, essentially as specified by the above-mentioned authors (3) for their Ointment No. 2.

Parenteral Solutions.—Sterile aqueous solutions containing 25 mg. of the maleate per milliliter, certain of which contained 0.25% phenol, were also assayed.

PROCEDURES

Tablets.—A solution containing 1 mg. of the maleate per ml. was prepared by allowing 10 tablets to disintegrate in approximately 100 ml. of distilled water and then adjusting the volume to 250 ml. for the 25-mg. tablets and to 500 ml. for the 50-mg. tablets. After thorough mixing, a 10-ml. aliquot was diluted to 500 ml. with distilled water to yield a solution containing ≃2 mg. of pyranisamine maleate per 100 ml., which was then assayed. Optical densities (*d*) of the diluted pyranisamine solutions were measured at 2435 Å. in the Beckmann spectrophotometer using 1-cm. cells, against a reference solution prepared by similar dilution of placebo tablets. The pyranisamine maleate content of the tablets was then calculated from the expression

$$\text{mg. pyranisamine maleate per tablet} = \frac{F \times d}{0.422}$$

where the factor F is 12.5 and 25.0, respectively, for 25- and 50-mg. tablets. The prescribed dilutions lead to a density (*d*) of ≃0.84. Lower densities are

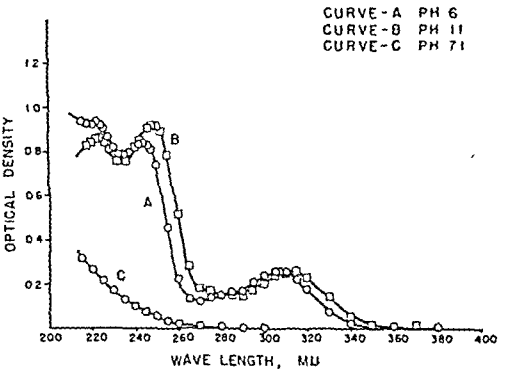

Fig. 2.—Absorption spectra of Pyranisamine Maleate (curves *A* and *B*; 2 mg./100 ml.) and Maleic Anhydride (curve *C*; 0.488 mg./100 ml.).

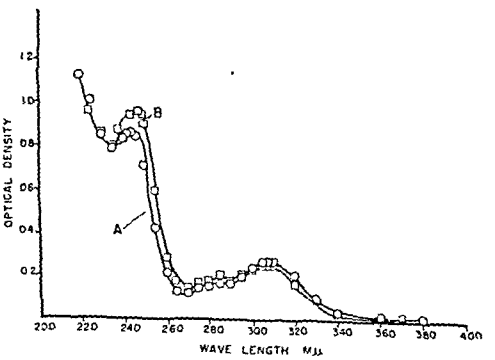

Fig. 3.—Absorption spectra of Pyranisamine Maleate in 20% alcohol (curve *A*; 2 mg./100 ml.) and in 1/1 Petroleum Ether-Absolute Alcohol (curve *B*; 2 mg./100 ml.).

obtained at greater dilutions, which may be chosen to suit the convenience of the operator. For greater dilution than prescribed above, the factor F must be appropriately increased. The same applies to the succeeding preparations.

The placebo tablets were fabricated in order to correct for light absorption or scattering by inert ingredients present. Actually it was observed that,

at the dilutions employed, these effects were inconsequential so that water served as a satisfactory reference liquid. This simplification cannot be taken for granted, however, in all cases; and each new composition must be examined for transparency before placebo tablets are discarded. Another precaution which must always be observed is the maintenance of a final solution *p*H of < 6.5.

Even when interference by turbidity occurs, placebo tablets can frequently be dispensed with simply by filtering the diluted unknown solution through a sintered glass filter prior to measurement of optical density. Filtration through paper, however, is not permissible because of absorption by the filter paper.

Elixirs.—The 0.5% elixirs were diluted 250 times with 20% alcohol or distilled water to a theoretical content of 2 mg. of maleate per 100 ml. of solution. The blank elixir was identically diluted to produce the reference solution needed for the spectrophotometric measurements. Optical densities (*d*) were determined as described above for tablets, and elixir concentrations calculated from

$$\text{mg. pyranisamine maleate per ml. of elixir} = \frac{2.5d}{0.422}$$

In certain instances the light absorption by components (especially the essential oils) was so great as to overshadow the absorption by the maleate proper. I nsuch cases, the required precision of preparation of reference solutions became a burden and could not always be attained. This difficulty was eliminated by removing the interfering components of the elixirs in the following manner. Ten milliliters of elixir was diluted with distilled water in a separatory funnel to 100 ml. and shaken with three successive 10-ml. portions of reagent ethyl ether, the organic solvent being removed after each extraction. The remaining aqueous phase was then further diluted (total dilution of 250 times) with distilled water to yield a solution containing 2 mg. of the antihistamine compound per 100 ml. Once the absorbing materials have been extracted, the elixir blank may be discarded and the measurement of optical density may be made against water as reference liquid.

Anhydrous Petrolatum Ointments.—A 5-Gm. sample of the 5% ointments was dissolved in 100 ml. of petroleum ether, and extracted with five 25-ml. portions of distilled water. These combined aqueous extracts containing the pyranisamine maleate were diluted with distilled water to 500 ml. and, after thorough mixing, a 10-ml. aliquot was then adjusted to 250 ml. with distilled water. This solution, containing ≃2 mg. of the antihistamine compound per 100 ml., was then utilized for optical density (*d*) measurements against distilled water as reference liquid, as described above. The maleate content can then be calculated from the expression

$$\text{mg. pyranisamine maleate per Gm. of ointment} = \frac{25d}{0.422}$$

Anhydrous Water-Soluble Ointments.—A 5-Gm. sample of the 5% ointment was dissolved in distilled water to make 500 ml. of solution, after which a 10-ml. aliquot was further diluted to 250 ml., yielding a solution of ≃2 mg. pyranisamine salt per 100 ml. The reference solution was prepared solution of 4.75 Gm. of ointment base and dilution described for the ointment itself. From the dens (*d*) at 2430–2435 Å. of the ointment solution agai the blank, the maleate content was calculated follows:

$$\text{mg. pyranisamine maleate per Gm of ointment} \quad \frac{2}{0.4}$$

Aqueous Emulsion-Type Ointments.—A 5-G sample was dissolved and diluted precisely as d scribed for the preceding type of ointment, exce that the solvent in this case was a mixture of equ volumes of absolute ethyl alcohol and petroleu ether. The reference solution was obtained l similar solution and dilution of 4.75 Gm. of the bla ointment base. In this case, however, the optic density (*d*) was measured at 2475 Å., and the co centration of maleate calculated from

$$\text{mg. pyranisamine maleate per Gm. of ointment} \quad \frac{2}{0.47}$$

Parenteral Solutions.[1]—The solutions containi 25 mg. of maleate per milliliter were diluted 12 times to yield a solution containing ≃2 mg./1 ml. of which the optical density (*d*) at 2435 Å. w determined against water since the phenol did n interfere. Concentration was calculated from

$$\text{mg. pyranisamine maleate per ml. of solution} \quad \frac{12.}{0.4}$$

DISCUSSION OF RESULTS

The usefulness of the method is indicated by typical assay results compiled in Table III which gives in succession the type, number, and history of a preparation, the medium in which the light absorption was measured, the theoretical maleate content, the amount as determined spectrophotometrically, and finally, the per cent deviation from theory. Scrutiny of the last column reveals that only one assay result differs from the expected composition by more than 10%. In most cases, deviations are 5% or less.

The heat and storage stability[2] of certain of these preparations is indicated by the data in Table III. The parenteral solutions, for example, appear to withstand autoclaving for twenty minutes at 120°, as well as subsequent storage for six months at room temperature and 40°, without suffering a loss of pyranisamine content as judged by this method of assay. Similar experiments with elixirs reveal a constant potency even after a year of storage at 40°. Thus the spectrophotometric assay method served to determine both the potency and the stability of these preparations.

SUMMARY

1. A spectrophotometric method of assaying for pyranisamine maleate in pharmaceutical

[1] Prepared by Mr. T. J. Macek of this Laboratory.

[2] The conclusion as to stability is based on the invariance of the ultraviolet absorption spectrum. It is reasonable to assume that, for this type of molecule, any significant degree of decomposition would be attended by measurable changes in absorption spectrum.

TABLE III.—ASSAY RESULTS

Preparation		Storage			Pyranisamine Maleate Content		
Type	No.	Time	Temp.	Medium	Potency	Assay	% Deviation
Elixir	1	Fresh		20% Alcohol	5 mg./ml.	4.90 mg./ml.	− 2.0
	1	Fresh		20% Alcohol	5 mg./ml.	5.29 mg./ml.	+ 5.8
	1	2 mo.	Room	20% Alcohol	5 mg./ml.	5.12 mg./ml.	+ 2.4
	1	2 mo.	40°	20% Alcohol	5 mg./ml.	5.00 mg./ml.	0.0
	1	3 mo.	Room	20% Alcohol	5 mg./ml.	5.18 mg./ml.	+ 3.6
	1	3 mo.	40°	20% Alcohol	5 mg./ml.	5.12 mg./ml.	+ 2.4
	1	6 mo.	Room	Distilled water	5 mg./ml.	5.05 mg./ml.	+ 1.0
	1	6 mo.	40°	Distilled water	5 mg./ml.	4.85 mg./ml.	− 3.0
	1	12 mo.	Room	Distilled water	5 mg./ml.	5.10 mg./ml.	+ 2.0
	2	Fresh		20% Alcohol	5 mg./ml.	4.87 mg./ml.	− 2.6
	3	Fresh		Distilled water	5 mg./ml.	5.00 mg./ml.	0.0
	4[a]	Fresh		Distilled water	5 mg./ml.	5.05 mg./ml.	+ 1.0
	5[a]	Fresh		Distilled water	5 mg./ml.	5.00 mg./ml.	0.0
	6[a]	Fresh		Distilled water	5 mg./ml.	5.00 mg./ml.	0.0
Tablet	1	...	...	Distilled water	25 mg./tab.	23.5 mg./tab.	− 6.0
	2	...	...	Distilled water	25 mg./tab.	23.0 mg./tab.	− 8.0
	3	...	...	Distilled water	50 mg./tab.	48.5 mg./tab.	− 3.0
	4	...	...	Distilled water	50 mg./tab.	47.5 mg./tab.	− 5.0
	5	...	...	Distilled water	50 mg./tab.	46.5 mg./tab.	− 7.0
	6	...	...	Distilled water	50 mg./tab.	52.0 mg./tab.	+ 4.0
	7	...	...	Distilled water	50 mg./tab.	52.5 mg./tab.	+ 5.0
	8	...	...	Distilled water	50 mg./tab.	49.0 mg./tab.	− 2.0
	9	...	...	Distilled water	50 mg./tab.	47.0 mg./tab.	− 6.0
	10	...	...	Distilled water	50 mg./tab.	47.5 mg./tab.	− 5.0
	11	...	...	Distilled water	50 mg./tab.	48.5 mg./tab.	− 3.0
	12	...	...	Distilled water	50 mg./tab.	51.5 mg./tab.	+ 3.0
Petrolatum ointment	1	...	...	Distilled water	50 mg./Gm.	54.5 mg./Gm.	+ 9.0
	2	...	...	Distilled water	50 mg./Gm.	46.5 mg./Gm.	− 7.0
	3	...	...	Distilled water	50 mg./Gm.	51.0 mg./Gm.	+ 2.0
Water-soluble ointment	1	...	...	Distilled water	50 mg./Gm.	51.0 mg./Gm.	+ 2.0
	2	...	...	Distilled water	50 mg./Gm.	48.5 mg./Gm.	− 3.0
	3	...	...	Distilled water	50 mg./Gm.	50.5 mg./Gm.	+ 1.0
Emulsion-type ointment	1	...	...	1:1 Absolute alcohol–petroleum ether	50 mg./Gm.	47.0 mg./Gm.	− 6.0
	2	...	...	1:1 Absolute alcohol–petroleum ether	50 mg./Gm.	56.0 mg./Gm.	+12.0
Parenteral solution	1[b]	Fresh		Distilled water	25 mg./ml.	24.9 mg./ml.	− 0.4
	2[c]	Fresh		Distilled water	25 mg./ml.	25.3 mg./ml.	+ 1.2
	3[b]	Fresh		Distilled water	25 mg./ml.	24.3 mg./ml.	− 2.8
	3[b]	6 mo.	Room	Distilled water	25 mg./ml.	25.1 mg./ml.	+ 0.4
	3[b]	6 mo.	40°	Distilled water	25 mg./ml.	25.0 mg./ml.	0.0
	4[c]	Fresh		Distilled water	25 mg./ml.	24.7 mg./ml.	− 1.2
	4[c]	6 mo.	Room	Distilled water	25 mg./ml.	25.0 mg./ml.	0.0
	4[c]	6 mo.	40°	Distilled water	25 mg./ml.	24.2 mg./ml.	− 3.2

[a] Diluted elixir extracted with ethyl ether prior to assay.
[b] Autoclaved 20 min. at 120°.
[c] Contained 0.25% phenol.

preparations is described. This method is based on the ultraviolet absorption spectrum of the compound.

2. The method has been applied to elixirs, tablets, parenteral solutions, and several types of ointments. In a number of cases, assay results deviate from the theoretical content by less than 5 per cent.

3. The stability of certain preparations containing pyranisamine maleate has been established spectrophotometrically.

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Studies on Pharmaceutical Powders and the State of Subdivision. III. An Evaluation of Pharmaceutical Dispensing Techniques Employed to Increase the Specific Surface Areas of Powders*, †

By J. V. SWINTOSKY‡, S. RIEGELMAN,§ T. HIGUCHI, and L. W. BUSSE

The low-temperature nitrogen adsorption technique was applied to specific surface area determinations on pharmaceutical powders in experiments attempting to evaluate certain comminutative methods used in dispensing practice. Separate samples of zinc oxide, bismuth subnitrate, and sulfanilamide were levigated according to standard techniques with a mortar and pestle, a muller, and a spatula. Their specific surface areas were then determined. Data are presented which indicate the relative efficacy of these comminutative methods in reducing the surface average particle size of these several powders.

No quantitative studies of the relative efficacies of the common comminutative procedures employed by dispensing pharmacists appear to have been made in the past. Because of the lack of sensitivity to minor changes in particle size of the previously available methods of analysis,[1] such investigations were necessarily restricted mainly to qualitative observation. A study of this nature, employing the relatively precise nitrogen adsorption technique, is presented.

EXPERIMENTAL

Powders Studied.—The powders chosen for this analysis were U. S. P.[2] Zinc Oxide, U. S. P. Bismuth Subnitrate, and U. S. P. Sulfanilamide. They represent lots of powders with surface average particle sizes of approximately 0.3 μ, 0.6 μ, and 14 μ respectively, as shown by measurements with a low-temperature nitrogen adsorption apparatus (1, 2).

Equipment.—The dispensing equipment used for these studies was new and of conventional type. A 6-inch porcelain mortar was employed. The ground glass muller measured 3 inches across the base, and a 6-inch stainless steel spatula was used. The pill tile used in conjunction with the muller and spatula measured 12 inches square, and all operations of levigation were performed on the ground glass half of the pill tile surface.

Procedure.—Levigation was performed both on the dry materials and on the water pastes of these materials. When water was used, it was added in increments to maintain a desirable consistency of the paste, as one might do in a dispensing operation.

Certain arbitrary procedures were adopted as a means of comparison of these dispensing techniques. One revolution of the pestle, one revolution of the muller, and one sweep with the spatula were all tacitly assumed to require equal physical effort. A single revolution of the pestle consisted of one complete circular movement in the mortar. A single revolution with the muller consisted of one complete circular movement over the ground glass surface of the pill tile. A single sweep with the spatula consisted of a forward motion across the ground glass surface of the pill tile culminated by a backward motion bringing the spatula back to its original starting position. Table I outlines the conditions employed in evaluating the effects of these several dispensing techniques.

After levigation, all powders were dried in a desiccator over anhydrous magnesium perchlorate for a period of at least three weeks. The masses were then crushed to a granular consistency[3] and the specific surface areas were measured with a nitrogen adsorption apparatus as described in previous papers (1, 2) of this series.

RESULTS AND DISCUSSIONS

The results of the measurements on the three pharmaceutical powders after various pretreatment are illustrated in Table II and Figs. 1–3. The effects of the several comminutative methods are seen to differ for the different powders. Zinc oxide did not show an increase in surface area by any of the techniques employed. The specific surface values obtained after pretreatment agreed with the value for the untreated powder. However, bismuth subnitrate showed a marked relative increase in specific surface area with all of the comminutative methods used. Sulfanilamide also appeared to be easily reduced in particle size by all of these methods.[4]

Results on analysis of these few powders would appear to indicate that the extent of specific surface

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† This paper is based in part on a thesis submitted by Joseph V. Swintosky to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[1] Such as, for example, sieving, sedimentation analysis, etc.

[2] U. S. P. grade chemicals imply U. S. P. XII or XIII.

[3] A mortar and pestle was used to crush the hard masses into granular fragments. It was not necessary to grind the granules to the same condition as the original powder since the nitrogen gas penetrates the granules and measures the absolute surface area as well when the powder is aggregated as when it exists in the nonaggregated condition.

[4] It was observed in the course of these dispensing operations that the bulk volumes of both the zinc oxide and the bismuth subnitrate were reduced to about three-fourths of their original volumes. This observation was not made with the sulfanilamide.

TABLE I.—DESCRIPTION OF COMMINUTATIVE TECHNIQUES EMPLOYED ON SEVERAL PHARMACEUTICAL POWDERS

Powder	Implement	Gm. of Powder Levigated during One Complete Operation	Levigating Medium	Quantity of Liquid, Cc.	Number of Revolutions or Sweeps	Approximate Levigating Time, Min.
Zinc Oxide[a] Sample No. 2A	Mortar and Pestle	25	None	None	300	6
	Mortar and Pestle	25	Water	10	300	6
	Spatula	25	Water	13	300	6
	Muller	25	Water	13	300	6
Bismuth Subnitrate,[a] Sample No. 4A	Mortar and Pestle	23	None	None	300	6
	Mortar and Pestle	23	Water	7	300	6
	Spatula	23	Water	9	300	6
	Muller	23	Water	9	300	6
Sulfanilamide,[a] Sample No. 6A	Mortar and Pestle	10	None	None	300	6
	Mortar and Pestle	10	Water[b]	8	300	6
	Spatula	10	Water[b]	14	300	6
	Muller	10	Water[b]	12	300	6

[a] Analyses of these untreated samples have been reported in Papers I and II of this series.
[b] In levigation of the sulfanilamide pastes, the water used was previously saturated with sulfanilamide.

TABLE II.—SURFACE AREA MEASUREMENTS OF PHARMACEUTICAL POWDERS BY NITROGEN ADSORPTION AFTER TREATMENT BY SEVERAL DISPENSING TECHNIQUES

Material	Method of Treatment	Weight of Sample, Gm.	Sample Number	Analysis Number	V_m, Cc.	Area, M^2/Gm.	Average Area, M^2/Gm.	Density	Surface Average Diameter of Particle, Microns
Zinc Oxide (U. S. P.)	Untreated		2A (see Table I)				3.56	5.47	0.308
	Dry mortar						3.53	5.47	0.311
		21.26		1	0.806	3.51			
		21.26		2	0.814	3.54			
	Wet mortar						3.51	5.47	0.313
		21.32		1	0.806	3.51			
		21.32		2	0.806	3.51			
	Muller						3.52	5.47	0.312
		21.95		1	0.810	3.52			
		21.95		2	0.810	3.52			
	Spatula						3.51	5.47	0.313
		21.06		1	0.806	3.51			
		21.06		2	0.806	3.51			
Bismuth Subnitrate (U. S. P.)	Untreated		4A (see Table I)				0.63	4.93	1.93
	Dry mortar						1.57	4.93	0.775
		19.72		1	0.363	1.58			
		19.72		2	0.362	1.57			
	Wet mortar						0.89	4.93	1.37
		21.27		1	0.204	0.887			
		21.27		2	0.206	0.896			
	Muller						0.82	4.95	1.48
		20.61		1	0.188	0.820			
		20.61		2	0.188	0.820			
	Spatula						0.73	4.93	1.67
		22.28		1	0.169	0 735			
		22.28		2	0.167	0.726			
Sulfanilamide (U. S. P.)	Untreated		6A (see Table I)				0.28	1.49[a]	14.4
	Dry mortar						0.46	1.49	8.75
		31.84		1	0.106	0.461			
		31.84		2	0.105	0.457			
	Wet mortar						0.61	1.49	6.60
		31.70		1	0.142	0.618			
		31.70		2	0.137	0.596			
	Muller						0.45	1.49	8.95
		29.35		1	0.106	0.461			
		29.35		2	0.102	0.444			
	Spatula						0.40	1.49	10.1
		29.34		1	0.093	0.408			
		29.34		2	0.091	0.399			

[a] Density determined with nitrogen adsorption apparatus as described in Paper II of this series.

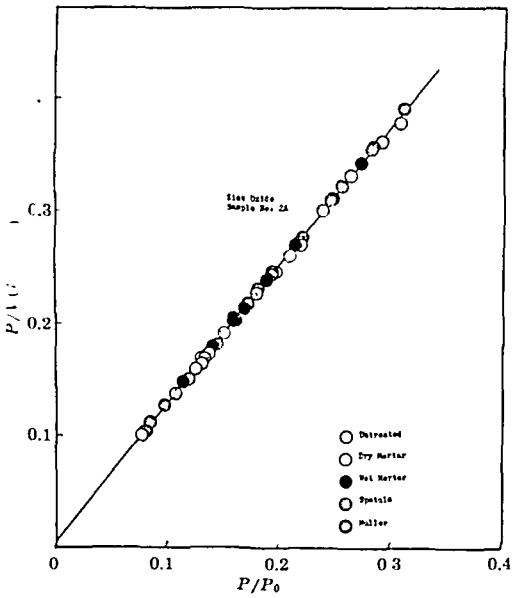

Fig. 1.—BET plots for nitrogen on untreated zinc oxide and on the same zinc oxide treated according to the dispensing techniques indicated. This sample of zinc oxide has previously been reported in Paper I of this series.

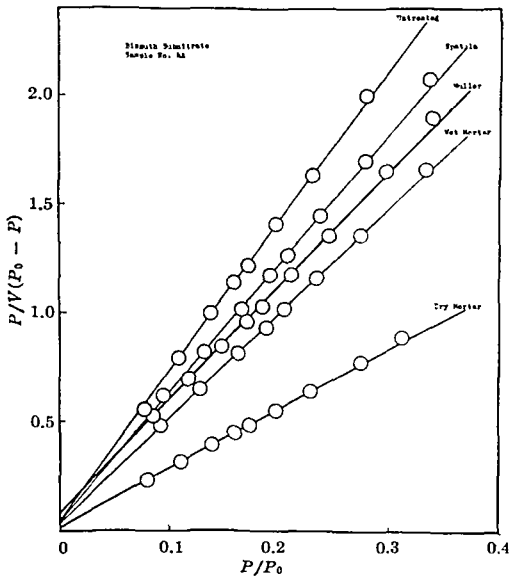

Fig. 2.—BET plots for nitrogen on untreated bismuth subnitrate and on samples of bismuth subnitrate treated according to the dispensing techniques indicated. This sample of bismuth subnitrate has previously been reported in Paper II of this series.

area increase is not influenced by the comminutative technique alone, but probably is also dependent upon certain physical characteristics of the powder such as hardness, brittleness, friability, and initial particle size. The limited data point to the mortar and pestle as the implements of choice where redu tion of particle size is desirable. Where only a mixi effect is desired or where aggregates only are to reduced, the convenient spatula technique wou be satisfactory; however, its use for the purpose increasing the specific surface area of powders duri incorporation in a liquid or semisolid base would n seem to be the best procedure.

From this preliminary study it would appear th the mortar and pestle should be used to effect maximum reduction in particle size prior to i corporation of a powder in another medium such an ointment base.

SUMMARY

A preliminary investigation is presented on t relative efficacy of the mortar and pestle, t

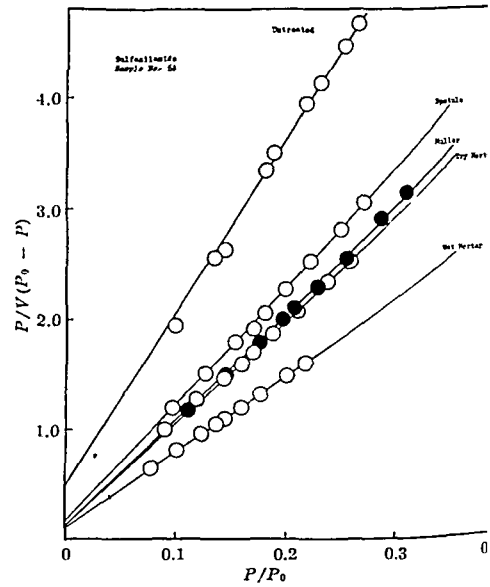

Fig. 3.—BET plots for nitrogen on untreated su fanilamide and on samples of sulfanilamide treate according to the dispensing techniques indicate This sample of sulfanilamide has previously bee reported in Paper II of this series.

muller, and the spatula as comminutativ methods in certain dispensing operations. Th powders chosen for this analysis were U. S. I Zinc Oxide, U. S. P. Bismuth Subnitrate, an U. S. P. Sulfanilamide. The specific surface are measurements were made with a nitrogen ad sorption apparatus.

The particle size of zinc oxide was not affecte by any of these comminutative methods under th conditions of the experiment. Both bismut subnitrate and sulfanilamide were reduced i particle size by levigation. The mortar an pestle was most effective and the spatula wa least effective in reducing the particle size of th latter two powders.

The results would appear to indicate that the extent of particle size reduction is not influenced by the comminutative technique alone, but also by certain physical properties of the powder.

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Microtoxicology. V. Colorimetric Reactions and Optical Crystallographic Properties of Several Antihistaminics of Unrelated Structure*,†

By THOMAS J. HALEY‡ and GEORGE L. KEENAN§

A scheme, based upon colorimetric and precipitation reactions, has been devised for the identification and differentiation of several antihistaminic drugs. The tests employed are described and the results obtained are tabulated.

A RECENT REVIEW (1) showed that although much was known regarding the pharmacological actions of Thephorin (2-methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene), Sc 887 (diethylaminoethyl - 9,10 - dihydroanthracene - 10-carboxylate), 1571F (N′-phenyl-N′-ethyl-N-diethylethylenediamine) and 929F (2-isopropyl-5-methylphenoxyethyldiethylamine), no means was available for their rapid identification. This is also the case with the two new antihistaminics, Diatrin (N′-phenyl-N′-(2-thenylmethyl)-N-dimethylethylenediamine) (2, 3) and Trimeton (1-phenyl - 1 - (2 - pyridyl) - 3 - dimethylaminopropane) (4). However, information is available concerning some of the physical constants and derivatives of all of the above compounds (see Table I).

In view of the scarcity of information on methods for the identification and differentiation of these compounds, it was decided that they should be investigated in the same manner as the other antihistaminics (7–9). Further, so that a comparison of results could be made the methods employed previously were used for this evaluation.

* Received Mar. 17, 1949, from the Medical School, University of California at Los Angeles.
† The authors wish to thank Hoffmann-La Roche, Inc., for the Thephorin, Schering Corporation for the Trimeton, G. D Searle & Company for the Sc 887, Abbott Laboratories for the 929F and 1571F, and William R. Warner & Company for the Diatrin used in this work.
‡ Medical School, University of California at Los Angeles.
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EXPERIMENTAL

The reagents used were prepared as directed by Thienes and Haley (10). To one drop of reagent on a microscope slide or in the well of a spot plate was added about 1 mg. of the compound to be tested and the resulting precipitate or color observed for thirty minutes. Where definite crystalline precipitates were produced, they were allowed to dry and the crystals were then examined by the immersion method for their crystallographic characteristics. The end point with the acidic colorimetric reagents was a charring or the development of a stable color. Where charring occurred only the colors produced prior to this were considered. The results obtained with both types of reagent are given in Table II.

To aid further in the identification of these antihistaminic drugs, photomicrographs of their pure crystals were taken with a Visicam photographic attachment of a Bausch and Lomb binocular microscope using the 16-mm. objective and the 10× eyepiece. See Fig. 1, parts *a*, *b*, *c*, *d*, and *e*, for the photomicrographs.

Inasmuch as the precipitation and colorimetric tests were almost identical for each compound, the optical crystallographic properties of the pure compounds were studied. Trimeton could not be studied in this manner because it was a liquid and it did not form any characteristic crystalline precipitates. Thephorin, Sc 887, and 1571F could not be examined crystallographically due either to the minute size of the particles or to unsatisfactory orientation. This is illustrated in the photomicrographs.

929F HCl

In Ordinary Light.—The substance consists of very small colorless rods and needles.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is parallel and the sign of elongation positive.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—Because of the minute size of the crystals, interference figures are rare. Optic sign apparently (+).

TABLE I.—PHYSICAL CONSTANTS OF ANTIHISTAMINIC COMPOUNDS

Compound	Derivative	M. P., ° C.	B. P. ° C.	B. P. Mm.	Referenc
Thephorin	Hydrogen tartrate	165–167	....		4
Trimeton	Base	...	135	0.5	3
	Picrate	203–204.5	....		
Sc 887	HCl	170–171	....		5
929F	HCl	121–122	....		...
1571F	HCl	97.5–98.5	....		...
Diatrin	Base	...	183–185	7.0	6
	n_D^{25} 1.5902				
	HCl	186–187	....		

TABLE II.—COLORIMETRIC AND PRECIPITATION REACTIONS OF ANTIHISTAMINICS

Reagent	Trimeton	Thephorin	Sc 887	929F	1571F	Diatrin
Conc H_2SO_4	Colorless	Canary yellow	Deep orange-yellow	Yellow then pink	Orange	Orange-yello
Conc HNO_3	Canary yellow	Colorless	Very faint yellow	Deep blue	Lemon yellow then orange	Brownish red
Mandelin's	Colorless	Greenish blue, then deep blue	Faint greenish yellow	Pale brown with purple center	Bright orange	Orange-red changing t pale yellow
Marquis'	Colorless	Pale brown	Pale brown	Carmine	Pale yellow	Pale yellow
Fröhde's	Colorless	Greenish yellow	Greenish yellow to orange	Pale green then red-violet	Colorless	Orange
Buckingham's	Colorless	Olive green	Green to bluish green	Bluish green then red-brown to black	Colorless	Orange-red
Chloroplatinic acid	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous
Chloroauric acid	Amorphous	Amorphous	Amorphous	Square flat plates of varying sizes	Broad leaflike crystals. Reagent turns blue green	Amorphous
Picric acid saturated aqueous solution	Thin small needles in crosses and bundles	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous

Refractive Indices.—α = 1.549, β = 1.556, γ = 1.570; all ±0.002. All the indices are readily found. α usually occurs crosswise, β crosswise, and γ lengthwise.

Diatrin HCl

In Ordinary Light.—The substance consists of colorless, irregular fragments and elongated six-sided rods with obtuse terminations.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—Many of the fragments do not extinguish sharply with crossed nicols.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—Optic axis figures frequently are shown. Optic sign (−).

Refractive Indices.—α = 1.604, β = 1.675, γ = 1.733; all ±0.002. Most of the fragments show the α and β indices.

DISCUSSION

Although Trimeton may be differentiated from the other antihistamines by the use of concentrated sulfuric acid, the test cannot be considered specific because such alkaloids as quinine and atropine give a similar reaction with this acid (11). The reactions given by the other compounds investigated with the exception of 929F are very similar to those reported for Benadryl (8), narcotine, solanine, thebaine, and veratrine (11) and it would be difficult to distinguish between the colors observed. However, the reaction between concentrated sulfuric acid and 929F differs in the final color produced from any recorded in the literature so that the acid would aid in differentiating this antihistaminic from the other compounds.

Concentrated nitric acid produces the same color reaction with Trimeton and Sc 887 as it does with narceine, narcotine, physostigmine, strychnine, thebaine, and veratrine so that the test is of little value. This is also the case with 1571F in that codeine, morphine, and papaverine (11) as well as Tagathen (9) produce a yellow then red color with concentrated nitric acid. The test would be of value in distinguishing Diatrin from the other compounds. However, berberine gives a similar red-brown color but the reaction of the two compounds with sulfuric acid would serve to differentiate between them. The color produced by 929F and nitric acid differs from any reported in the literature and would be valuable as a further means of identification.

Thephorin reacts with Mandelin's Reagent similarly to papaverine and quinidine, the final color being identical with that produced by papaverine. However, other tests would differentiate between these two compounds. Mandelin's Reagent produces varying shades of green with berberine, codeine, colchicine, and coniine which are similar to that produced by Sc 887. However, the latter compound shows a stable color while the former compounds have varying final colors. The reaction between 929F and Mandelin's Reagent is sufficiently different from those reported for aconitine and digitalin (11) and Pyribenzamine (8) that it would serve as an additional confirmatory identification test. Both 1571F and Diatrin produce colors with Mandelin's Reagent which are very similar to those seen

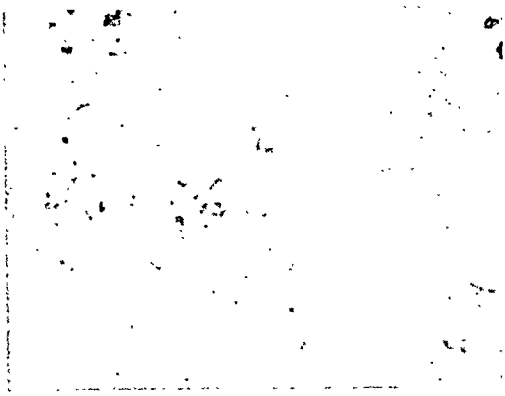

Fig. 1 (*a*).—Thephorin.

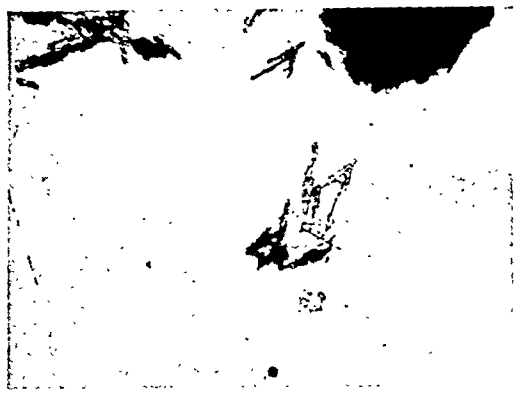

Fig. 1 (*d*).—929F.

Fig. 1 (*b*).—Sc 887.

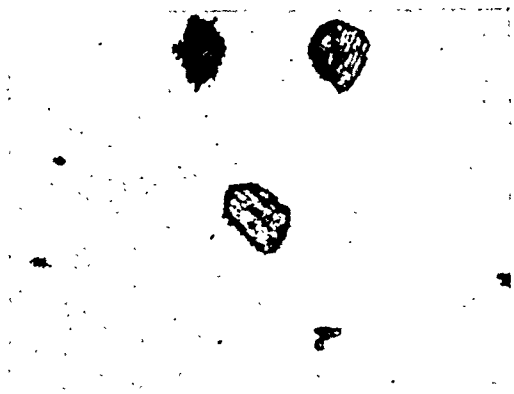

Fig. 1 (*e*).—Diatrin.

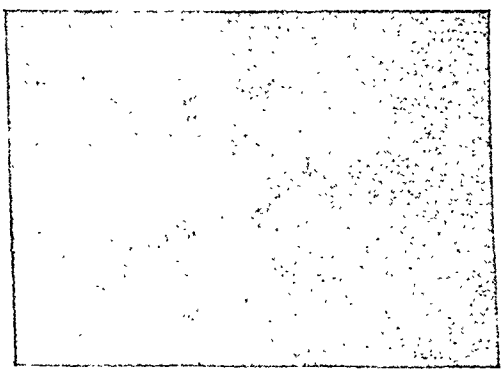

Fig. 1 (*c*).—1571F.

with the halogenated thenyl antihistamines (9) as well as cinchonine, cocaine, pilocarpine, quinine, solanine, thebaine, and veratrine so that the other tests given in Table II would be required before their positive identification was accomplished.

Marquis' Reagent is of value in differentiation of 929F from the other antihistaminic compounds tested (8, 9) and also from many alkaloids (11) because none of the compounds reported give a similar color reaction. However, it would be of but little value in differentiating Thephorin from Sc 887 and 1571F from Diatrin because of the color produced.

Many alkaloids (11) as well as Trimeton and 1571F do not react with Fröhde's Reagent so that this test was of little value. Further, the reactions of Diatrin and Benadryl (8) as well as brucine and solanine (11) are very similar so that differentiation can only be accomplished by observing the final colors produced in all cases. The final colors seen with Thephorin and Sc 887 differentiate these two compounds not only from each other but from colchicine (11). The reaction between 929F and Fröhde's Reagent differentiates it from the other antihistaminics and also from morphine which shows a final color of violet blue (11).

The reactions between Buckingham's Reagent and Thephorin and Sc 887 are similar to that seen with codeine, but other tests of these compounds would serve to differentiate them. The colors observed with both 929F and Diatrin differ from any previously reported (11).

Both chloroplatinic and picric acid are of little value as precipitation reagents with most of the compounds tested. However, Trimeton, which is nonreactive with most of the other reagents employed, gave a crystalline precipitate with picric

acid. The melting point of this picrate would serve as an identifying test for Trimeton.

The crystalline precipitates produced by the reaction between 929F and 1571F and chloroauric acid would serve as additional evidence of their identity because none of the other antihistaminics investigated (7–9) formed crystalline precipitates with this reagent.

SUMMARY

1. A scheme for the identification and differentiation of several antihistaminic drugs has been presented.

2. The colorimetric tests employed enable a rapid identification to be performed.

3. The precipitation reactions are most useful in identifying Trimeton, 929F, and 1571F.

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Microtoxicology. VI. Identification and Differentiation of the Newer Antihistaminic Drugs Related to Benadryl, Pyribenzamine, and Antergan*,†

by THOMAS J. HALEY‡ and GEORGE L. KEENAN§

Seven new antihistaminic drugs related to Benadryl, Pyribenzamine, and Antergan were tested with colorimetric and precipitation reagents and their optical crystallographic properties determined. Because of the similarity of the reactions, their crystallographic properties offer the best means of identification.

SINCE THE advent of Benadryl, Pyribenzamine, and Antergan, many new antihistaminic drugs have been synthesized and investigated both pharmacologically and clinically (1–13). Among these new compounds are Neoantergan (N′-*p*-methoxybenzyl - N′ - 2 - pyridyl - N - dimethylethylenediamine), Neoheteramine (N′-*p*-methoxybenzyl - N′ - 2 - pyrimidyl - N′ - dimethylethylenediamine), Linadryl (β-morpholinoethyl benzhydryl ether HCl), Dec apryn(α-(2-dimethylaminoethoxy)α(methylbenzyl)-pyridine), No. 204 (2-imidazoline-2-methyl benzhydryl ether), Antistine (N′-phenyl-N′-benzylaminomethyl imidazoline), and Dramamine, Sc 1694 (N-dimethylethyl benzhydryl ether-8-chlorotheophyllinate). Of these compounds, only No. 204 has been investigated by the usual toxicological methods (14). However, several of the compounds have their melting points recorded in the literature (see Table I).

Because of the lack of information concerning the reactions of these compounds, it was decided that they should be investigated in the same manner and with the same reagents that had been used to identify the other antihistaminic agents. Further, comparisons of the reactions observed with those recorded in the literature should be made to prevent any misinterpretation in their identification and differentiation.

EXPERIMENTAL

The melting points without references in Table I were determined using a Thiele tube; they are uncorrected.

The reagents used were prepared as directed by Thienes and Haley (17). To one drop of reagent on a microscope slide or in the well of a spot plate was added about 1 mg. of the compound to be tested and the resulting color or precipitate observed for thirty minutes. Where definite crystalline precipitates were produced, they were allowed to dry and the crystals were then examined by the immersion method for their crystallographic characteristics. The end point with the acidic colorimetric reagents was a charring or the development of a stable color.

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* Received April 4, from the Medical School, University of California at Los Angeles.
† The authors wish to thank Merck and Co. for the Neoantergan Maleate; Nepera Chemical Co., Inc. for the Neoheteramine HCl; G. D. Searle and Co. for the Dramamine; Wm. S. Merrell and Co. for the Decapryn Succinate; Parke, Davis and Co. for the Linadryl HCl; and Ciba Pharmaceutical Products, Inc. for the Antistine HCl and No. 204 HCl used in this study.
‡ Medical School, University of California at Los Angeles.
§ Formerly Microanalyst, Food and Drug Administration; present address: Strongsville, Ohio.

Where charring occurred, only the colors produced prior to this were considered. The results obtained with both types of reagent are given in Table II.

To aid further in the identification of these antihistaminic drugs, photomicrographs of their pure crystals were taken with a Visicam photographic attachment on a Band L binocular microscope using the 16-mm. objective and the 10 × eyepiece. See Fig. 1, parts *a*, *b*, *c*, *d*, *e*, *f*, and *g*, for the photomicrographs.

Inasmuch as the precipitation and colorimetric tests were very similar for each of the compounds, the optical crystallographic properties of the pure compounds were studied. Due either to the minute size of the particles or to unsatisfactory orientation, optical crystallographic data could not be obtained on Neoantergan Maleate, Dramamine, Decapryn Succinate and No. 204 HCl. This is illustrated by the photomicrographs.

Neoantergan Maleate

In Ordinary Light.—The habit is rod-shaped and irregular fragments.

Dramamine

In Ordinary Light.—The habit is mostly colorless platy material with occasional rods.

Preparation No. 204 Hydrochloride

In Ordinary Light.—The habit is rod-shaped and irregular fragments.

Antistine Hydrochloride

In Ordinary Light.—The habit is rod-shaped and irregular fragments.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The rods show parallel extinction and the sign of elongations is usually positive. Many fragments do not extinguish sharply with crossed nicols.

TABLE I.—PHYSICAL CONSTANTS OF ANTIHISTAMINICS

Drug	Derivative	M. P., ° C.	B. P. ° C.	B. P. Mm.	Reference
Neoantergan	Base	.....	165–173	0.07	..
	HCl	143–143.5	.....	..	15
	Maleate	100–101	.....	..	..
Neoheteramine	HCl	171–172	.....	..	..
Linadryl A-446	HCl	179–180	.....	..	..
Decapryn	Base	.....	145–153	0.4	..
	HCl	169–170	.....	..	16
	Succinate	98.5–99.5	.....	..	..
No. 204	Base	.....	102–103	..	..
	HCl	203–205	.....	..	14
	Picrate	205	.....	..	..
Antistine	HCl	234 d	.....	..	..
Dramamine Sc 1694	8-Chlorotheophylline	83–85	.....	..	..

TABLE II.—COLORIMETRIC AND PRECIPITATION REACTIONS OF ANTIHISTAMINIC DRUGS

Reagent	Dramamine	Neoheteramine	Neoantergan	Decapryn	Linadryl HCl A 446	Antistine	No. 204
Conc. H_2SO_4	No change	Red	Magenta	Canary-yellow	Canary-yellow with oily orange-red droplets	Colorless	Bright orange
Conc. HNO_3	No change	Colorless	Colorless	Colorless	Colorless	Magenta	Colorless
Mandelin's	Cloudy bluish	Deep pink	Magenta	No change	No change	Brick-red	No change
Marquis'	Cloudy tan	Magenta	Magenta	No change	No change	No change	Pale yellow
Frohde's	No change	Magenta	Magenta	Canary-yellow then orange	Canary-yellow with oily orange-red droplets	Colorless	Canary-yellow with oily orange droplets
Buckingham's	No change	Magenta	Magenta	Canary-yellow then dirty green	Canary-yellow with oily orange-red droplets	Colorless	Canary-yellow with oily orange drops
Chloroplatinic acid	Amorphous	Amorphous	Amorphous then oily globules	Leaf-like agglomerates in bundles	Amorphous	Amorphous	Amorphous
Chloroauric acid	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous
Picric acid sat. aq. soln.	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous

Fig. 1 (*a*).—Neoantergan Maleate.

Fig. 1 (*d*).—Antistine HCl.

Fig. 1 (*b*).—Dramamine.

Fig. 1 (*e*).—Linadryl HCl.

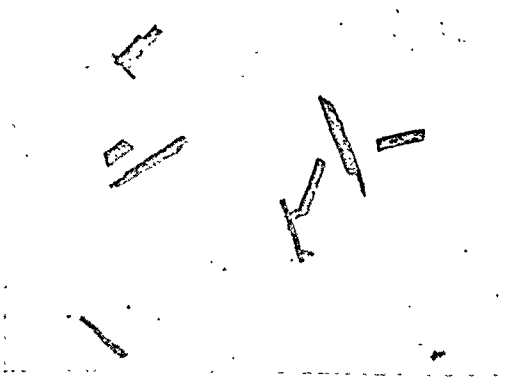


Fig. 1 (*c*).—Preparation No. 204.

Fig. 1 (*f*).—Neoheteramine HCl.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The substance is biaxial and acute bisectrix and optic axis figures are commonly found. 2E is not large. Optic sign (+).

Refractive Indices.—α = 1.683, β = 1.650, γ = >1.688 but <1.733; all ±0.002. α occurs crosswise on rods, β lengthwise, and the value 1.688 crosswise on rods. β is most commonly found.

Linadryl Hydrochloride

In Ordinary Light.—The substance consists of elongated, six-sided, and irregular fragments.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The fragments invariably extinguish sharply with crossed nicols. Extinction parallel and inclined on elongated forms. The elongation is usually positive.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The fragments extinguishing sharply with crossed nicols, interference figures are rarely shown. Optic sign apparently (−).

Refractive Indices.—$\alpha = 1.577$, $\beta = 1.631$, $\gamma = 1.672$; all ± 0.002. α common on rods showing parallel extinction (shown crosswise) and β lengthwise on rods showing parallel extinction. α and β are most commonly found.

Neoheteramine Hydrochloride

In Ordinary Light.—The substance is mostly colorless platy material with occasional rods.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The plates invariably extinguish sharply with crossed nicols. The elongated forms show parallel extinction and positive elongation.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The sharp extinction with crossed nicols precludes ideal interference figures. Optic sign apparently (−).

Refractive Indices.—$\alpha = 1.612$, $\beta = 1.679$, $\gamma = 1.691$; all ± 0.002. α and β are the most frequent values shown on plates lying horizontally. The γ value occurs much less frequently.

Fig. 1 (g).—Decapryn Succinate.

DISCUSSION

It is difficult to identify Dramamine with any of the reagents used with the exception of Mandelin and Marquis. The colorimetric reactions with these reagents differ from those reported for the alkaloids (18) and the other antihistaminic drugs (19–21). This may have been due to the 8-chlorotheophyllin part of the molecule obscuring or preventing the usual reactions of the Benadryl part (19).

Neoheteramine's reaction with concentrated sulfuric acid is similar to that of Benadryl (19) but the presence of the oily red globules in the latter's reaction makes differentiation easy. The final colors of Histadyl also are sufficiently different so that no mistake can be made in differentiation of the two compounds. Some difficulty might be encountered with curarine, veratrine, solanine, and thebaine (18), but the reactions of these alkaloids with the other reagents used enable an identification of the exact compound to be made. Neoheteramine's reaction with Mandelin's reagent differs from any previous report so that this reaction is the best means for colorimetric identification and differentiation. Marquis' reagent reacts with Neoheteramine to produce the same color shown by Neoantergan and Tagathen (20). With both Fröhde's and Buckingham's reagents, the colors given by Neoheteramine and Neoantergan are the same but they differ from those previously reported. Furthermore, no confusion should result because the other reagents react differently with these two antihistaminics.

Neoantergan reacts with concentrated sulfuric acid similarly to Bromothen, Chlorothen, and Tagathen but the final colors of these latter compounds are considerably different from that produced by Neoantergan. The fact that Neoantergan produced the same color regardless of the reagent used makes its identification very difficult.

Decapryn gives the same reactions with concentrated sulfuric and nitric acids as Thephorin (21) but the two compounds react very differently with the other reagents so that no confusion should result in their differentiation. The reactions of Decapryn, Linadryl, No. 204, and Benadryl (19) with Fröhde's and Buckingham's reagents are very similar, differing only in the final colors produced. However, in the case of Decapryn the greenish color observed with Buckingham's reagent would serve to differentiate this drug from the others. The crystalline precipitate produced when Decapryn was added to chloroplatinic acid is very similar to that seen with Histadyl (20) but the colorimetric reactions of the two compounds are so different that one could not be mistaken for the other.

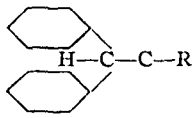
As pointed out previously, the reactions of Linadryl, No. 204, and Benadryl (19) with Fröhde's and Buckingham's reagents are identical. Furthermore, their reactions with concentrated sulfuric acid are sufficiently alike to make differentiation difficult. The structural similarity of the three compounds may be the reason for the identical colorimetric reaction.

From the structural formulas on Page 388, it would seem that the colorimetric reaction is due to the benzhydryl ether portion of the molecule, because this is the part of the molecule which is the same in all three compounds. Furthermore, when this part of the molecule is changed by the substitution of a pyridyl group for a phenyl group, as in Decapryn, the color produced is different.

The colorimetric reaction of Antistine with concentrated nitric acid differs from those of the other antihistaminics (19–21) and the alkaloids (18), and thus can be used for identification purposes. Antistine's reaction with Mandelin's reagent differs from those reported for the other antihistaminics but is similar to the reactions of atropine, burcine, morphine, narceine, narcotine, picrotoxin, solanine, thebaine, and veratrine. However, observation of the preliminary as well as the final colors will aid in differentiating these alkaloids from Antistine. In addition, use of the other tests will enable the toxicologist to identify the exact compound.

Although Cavallini and Mazzucchi (14) reported precipitation reactions between No. 204 and ammonium molybdate, picric acid, mercuric chloride, chloroauric acid, Mayer's reagent, chloroplatinic acid, and Reinecke's salt, only those precipitation reagents shown in Table II were investigated, because amorphous precipitates are of little value for identification purposes. It should be pointed out

also that Benadryl and Pyribenzamine have previously been shown to form similar amorphous precipitates with most of the reagents listed above (19). However, results in Table II confirm those of Cavallini and Mazzucchi (14).



Drug	R
Benadryl	$-CH_2-CH_2-N(CH_3)_2$
Linadryl	$-CH_2-CH_2-N$ (morpholine ring)
No. 204	$-CH_2-C$ (imidazolidine ring, N, CH_2, NH, CH_2)

SUMMARY

1. A method for the identification and differentiation of seven new antihistaminic drugs related to Benadryl, Pyribenzamine, and Antergan has been presented. Where the crystals were of sufficient size, the optical crystallographic properties were the most useful means for positive identification.

2. The colorimetric tests reported are useful in classification but should be combined with melting-point determinations for accurate identification.

3. The precipitation reagents used were of little value due to the formation of amorphous precipitates. However, Decapryn Succinate does form a crystalline precipitate with chloroplatinic acid.

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Studies on Indian *Datura**

By R. CHATTERJEE† and J. K. LAHIRI

Certain *Datura* species from Bengal compare favorably in the per cent of alkaloids with the same species found elsewhere.

THE MOST commonly occurring Indian species of *Datura*, which is hitherto known as *D. fastuosa* L., is synonymous with *D. fastuosa* var. *alba* and *D. alba* Nees. *D. fastuosa* of Linnaeus and of other authors is now treated merely as a variety of the type, namely, *D. metel* Linn. var. *fastuosa* (Linn.), (1).

THE ALKALOID CONTENT

It has been found that either hyoscyamine or hyoscine is the main constituent of the species of

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Datura. Table I shows the different species of *Datura* with respect to their main alkaloid. In the tables the old nomenclature has, however, been continued to avoid confusion.

TABLE I.—CLASSIFICATION OF *Datura* SPECIES

Name of the Species	Locality of the Species Examined
A. Species of *Datura* with hyoscyamine as the main alkaloid	
1. *D. stramonium*	America (2), British Columbia (3), Norway (4), Germany (5), Switzerland (5), Egypt (6), South Africa (7), India (8), China (9), Shanghai (10), and New Zealand (11)
2. *D. stramonium* var. *inermis*	Poland (12)
3. *D. fastuosa*	Holland (13)
4. *D. metel*	Patiala (India, 8), Germany (24, 25)
5. *D. tatula*	South Africa (7) and Peru (19)
6. *D. sanguinea*	Bogota (S. America, 14) and Peru (19)
7. *D. quercifolia*	Germany (24)
8. *D. ferox*	(27)
B. Species of *Datura* with hyoscine as the main alkaloid	
1. *D. arborea* (*Brugmansia suaveolens or B. candida*)	Germany (24, 25), Peru (19), and Bogota (14)
2. *D. meteloides*	Europe (26)
3(*a*). *D. fastuosa* var. *flor. coerul. plen.*	Assam (India, 8), Europe (8), Germany (23)
3(*b*). *D. fastuosa* var. *flor. alba*	Bengal (India) and Philippines (15)
4. *D. alba* Nees	Philippines (15, 18), Formosa (16), and China (22)
5. *D. innoxia* Miller	Ohio (17)
6. *D. metel* (*D.* var. *fastuosa*)	Lyallpur (India, 8), Germany (25)

It is interesting to note that even in one or two isolated cases, the nature of the alkaloid changed with respect to place; for instance, seeds of *D. metel* from Patiala (8) yielded hyoscyamine, although they usually produced hyoscine; and again *D. fastuosa* which is known to contain hyoscine had been found to contain hyoscyamine in Holland (13). The case of *D. metel* is particularly noteworthy, since even in places which are not geographically distant from one another, e.g., Lyallpur and Patiala, *D. metel* has synthesized a different alkaloid, obviously from the variation of soil and climate.

Since *D. fastuosa* (which is now called *D. metel* var. *fastuosa*, samples of which were assayed by the present writers) grows wild in Bengal, a tropical part of India, it was thought that a chemical examination of the different parts of the plant might lead to finding an economic source of either hyoscyamine or hyoscine.

With due attention to botanical controversy (1) it has been found that both the white and purple flowering *D. fastuosa* (now called *D. metel* var. *fastuosa*) contain hyoscine as the main alkaloid and that the quantity of the alkaloid is more or less the same in both the varieties. The average total alkaloids in the seeds is 0.34 per cent of which 77 per cent is hyoscine. (See Table II.)

EXPERIMENTAL

The Material.—Both the purple and white flowering plants of *Datura metel* var. *fastuosa* were collected from different places in the suburbs of Calcutta and from other parts of Bengal.

Preparation of Material for Estimation of Total Alkaloids.—Different parts of the plants, roots, leaves, fruits, and seeds were air dried, finely powdered (40 mesh), and kept in a vacuum desiccator over anhydrous calcium chloride before subjecting each to alkaloidal assays.

Estimation of Alkaloids.—Thirty-gram portions of powdered and dried drug were macerated in a strong-walled stoppered bottle with 300 cc. of ether, and after shaking vigorously, 15 cc. of 10% ammonia was added. The mixture was frequently shaken for two hours. The ethereal solution was decanted off and after a short drying with anhydrous sodium sulphate, it was filtered; 200 cc. of the filtered ethereal solution (i.e., 20 Gm. of drug) was evaporated to a few cc. on a water bath, 10 cc. of 0.5% hydrochloric acid was added, and the mixture was warmed again until the smell of ether disappeared. The acid solution was filtered into a separating funnel; the flask and the filter were washed thrice, each time with 5 cc. of water; the solution was then made slightly alkaline by carefully adding a freshly prepared saturated solution of sodium bicarbonate. The solution was extracted thrice, each time with 20 cc. of ether. The aqueous solution (*A*) containing hyoscyamine was put aside.

The ethereal solution was evaporated on the water bath to a few cc. Ten cubic centimeters of 0.5% hydrochloric acid was added and the mixture was warmed until the smell of ether disappeared. The acid solution was taken in a separating funnel, made alkaline with saturated solution of sodium carbonate as before, and extracted thrice with ether, each time with 20 cc. The aqueous solution still contained small quantities of hyoscyamine and was added to solution (*A*). The ethereal solution was treated as before, for a third time, and then dried with anhydrous sodium sulfate, evaporated on a water bath. The residue, which is hyoscine, was dissolved in 2 cc. of absolute alcohol and diluted with 10 cc. of water. The hyoscine was estimated by titration with decinormal hydrochloric acid with methyl red-methylene blue as indicator (1 cc. of 0.1 *N* HCl = 0.0303 Gm. hyoscine).

The combined aqueous solution (*A*) was treated with 3 cc. of concentrated ammonia and shaken thrice with chloroform, each time with 30 cc. The chloroform solution was shaken with 0.5 Gm. gum tragacanth, and after standing for about thirty minutes, it was filtered into a flask. The chloroform solution was evaporated completely on the water bath and the last traces of chloroform were removed by blowing air. The residue, hyoscyamine, was kept in a desiccator for several hours and then dissolved in 20 cc. of 90% alcohol. Ten cubic centi-

TABLE II.—THE PER CENT OF ALKALOIDS OF *D. fastuosa* (*D. metel* L. VAR. *fastuosa*) FROM DIFFERENT SOURCES

	Source	Parts Used	Total Alkaloid, %	Hyoscine, %	Hyoscyamine, %	Reference, %
1.	Europe	Seeds	0.200	0.100	0.100	(8)
2.	Germany					
	(*a*) Purple flowering	Seeds	0 254	0.220	0.034	(23)
	(*b*) White flowering	Seeds	0.223	0.200	0.023	(23)
3.	Holland	Seeds	0.149	...	0.149	(13)
4.	Philippines	Roots	0.248	...	...	(15)
		Leaves	0.221	...	...	
		Fruits	0.224	...	...	
		Seeds	0.443	...	...	
	White flowering	Roots and wood	0.170	...	...	(20)
		Leaves	0.210	...	...	
		Seeds	0.465	...	...	
	D. alba Nees	Leaves	0.210	...	...	(21)
		Fruits	0.175	...	...	
		Seeds	0.393	...	...	
5.	China					
	D. alba Nees	Flowers	0.485	...	...	(22)
6.	Formosa	Seeds	0.260	0.240	0.020	(16)
7.	Patiala (India)	Fruits	0 120	0.120	...	(8)
		Seeds	0.250	0.080	0.170	
8.	Lyallpur (India)	Leaves	0.250	0.250	Traces	(8)
		Seeds	0.230	0.230	Traces	
9.	Assam (India)	Roots	0.101	...	...	(8)
	Purple flowering	Leaves	0.119	...	...	
		Fruits	0.202	...	...	
10.	Bengal (India)					
	(*a*) Purple flowering	Roots	0.276	0.180	0.096	
		Leaves	0.143	0.091	0.052	
		Fruits	0.252	0.204	0.048	
		Seeds	0.383	0.288	0.095	
	(*b*) White flowering	Roots	0.240	0.151	0.089	
		Leaves	0.105	0.079	0.026	
		Fruits	0.223	0.192	0.031	
		Seeds	0.300	0.248	0.052	

meters of this solution were taken into a flask and diluted with 20 cc. of water. The alkaloid was estimated by titration with decinormal hydrochloric acid with methyl red-methylene blue as indicator (1 cc. of 0.1 *N* HCl = 0.02892 Gm. hyoscyamine).

The identity of both hyoscine and hyoscyamine was established by adding aqueous picric acid to the respective alkaloid solution after neutralization to obtain the picrates; then the crystals were purified by crystallizing twice from water. The picrate of hyoscine obtained had m. p. 186°, and that of hyoscyamine obtained had m. p. 163°. Pure hyoscine picrate has m. p. 188°, and hyoscyamine picrate has m. p. 165°.

SUMMARY

1. Both the purple and white flowering *D. metel* L. var. *fastuosa* (L.) contain approximately the same per cent of hyoscine and hyoscyamine in trace amounts.

2. The average total alkaloid in the seeds is 0.34 per cent, of which 77 per cent is hyoscine.

3. *Datura* species from Bengal compare favorably in the per cent of alkaloids with the same species found in other parts of India, e.g., Assam, Lyallpur, Patiala, and in different places—Japan, Philippines, Germany, and Holland.

4. It is recommended that the seeds of *D. metel* var. *fastuosa* may be used as a source of hyoscine.

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Determination of Theobromine in Theobromine and Sodium Salicylate*

By C. W. BELL†

A volumetric, acidimetric method is proposed as a substitute for the present N. F. gravimetric method. The proposed method features several advantages such as, ease of operation, saving of time, elimination of an empiric correction factor, and a high degree of precision and accuracy. Results are submitted to show that the present N. F. gravimetric method gives values which are erratic and usually high. A review of the literature on methods for the determination of theobromine is also included.

THE DETERMINATION of theobromine in theobromine sodium salicylate has been a question of controversy since the compound was first introduced to the medical profession in 1890 under the trade name "Diuretin." A wide variety of methods were proposed from time to time, were used for a while, only to be discarded for newer methods. A survey of the literature on the subject reveals that most of the earlier methods were of a gravimetric nature which simply involved dissolving the soluble salt in a small amount of water, precipitating the theobromine by adding acid, washing and weighing the residue without regard for the theobromine which might be dissolved in the wash water. When the compound was brought within the scope of the United States Pharmacopœia in 1916 (1) an improved gravimetric method was adopted as the official method. This method provided for the use of an empiric correction factor to compensate for the loss of theobromine. Many other attempts were made to standardize and improve the gravimetric method. In 1920 Schaap (2) suggested precipitation of the theobromine with zinc sulfate instead of one of the mineral acids. In 1921 Bennett and Windle (3) attempted to improve upon the United States Pharmacopœia IX method by more closely standardizing the conditions of the precipitation and the washing of the theobromine. They also proposed the use of a correction factor which would allow for a 14% loss in theobromine. Deér (4), in 1927, suggested the use of boric acid instead of a mineral acid for the precipitation of the theobromine and proposed a different correction factor. In the same year Mikó (5) carried out the precipitation in an ethyl alcohol, or ethyl alcohol-ether medium, and eliminated a correction factor. During this period when most of the investigators were presenting modifications of the gravimetric method, Emery and Spencer (6) announced an entirely new approach to the problem. This consisted of a volumetric method depending upon the formation of theobromine periodide, supposedly having the formula $C_7H_8O_2N_4 \cdot HI \cdot I_4$, by the action of iodine and potassium iodide upon a solution of theobromine in glacial acetic acid, and the determination of the excess of standard iodine with thiosulfate. The results by this method, however, do not show that it gives consistent results in the hands of various operators, and it has the very decided disadvantage that it would return as theobromine any substance which is capable of combining with iodine in acetic acid solution. To overcome these objections, as with the older gravimetric method, many modifications were introduced by a number of workers (7–12). None of these modifications were universally accepted as giving satisfactory and concordant results.

The first real important advancement in the determination of theobromine was the publication in 1930 of Boie's acidimetric method (13). This method is based on the fact that silver nitrate reacts with theobromine to form insoluble silver theobromine with the simultaneous quantitative liberation of nitric acid according to the following reaction: $C_7H_8O_2N_4 + AgNO_3 \rightarrow AgC_7H_7O_2N_4 + HNO_3$. The liberated nitric acid can then be titrated with standard alkali. The method has stood the test of time and has been slowly but en-

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† The author wishes to thank Mr. R. O. Hauck for his constructive comments in carrying out this study.

thusiastically received by a number of observers (14–17). The method now has official status through acceptance by the American Association of Official Agricultural Chemists for the determination of theobromine in theobromine calcium (18). In 1933, Mikó (19) proposed a method basically resembling the method of Boie in that an excess of standard silver nitrate is added to a solution of theobromine and sodium salicylate, the precipitated silver theobromine is filtered and the excess of silver nitrate determined in an aliquot portion of the filtrate with thiocyanate using ferric alum as an indicator. The accuracy of this method was confirmed by Zilberberg and Faĭnshteĭn in 1939 (20).

A review of the methods proposed for the determination of theobromine would not be complete without reference to two methods entirely different from the ones mentioned above: first, the methylation procedure reported by Self and Rankin (21) and later adopted as the official method of the British Pharmacopœia (22); second, a gravimetric method reported by Richardson and Campbell in 1942 (23) which utilizes the classical complex salt formation between theobromine and phosphotungstic acid. acid. When official recognition of Theobromine Sodium Salicylate was transferred from the United States Pharmacopœia to the National Formulary in 1942, the gravimetric assay method was not altered; in fact, the method of the present National Formulary (24) is essentially the same as when the drug first became official in 1916, although the method has long been acknowledged to be unsatisfactory due to the varying amounts of theobromine which are lost in the process. It has been the experience in these laboratories that more than ordinary care must be exercised in following the prescribed procedure in order to obtain consistently reproducible results; even if such results can be obtained there is always the likelihood of a constant error due to the fact that an empiric correction factor has to be used. A critical survey and investigation of practical methods for the determination of theobromine in the compound have led to the conclusion that of all the methods investigated, Boie's acidimetric method gives the fastest and most accurate results. The present study was undertaken in order to compare this method with the present N. F. VIII gravimetric method using the nitrogen content, as determined by Kjeldahl, as a standard of comparison.

EXPERIMENTAL

Ten commercial samples were used in this investigation, six of which were Theobromine and Sodium Salicylate N. F. VIII, made by two different manufacturers, and the other four were samples of "Diuretin." All of the samples were assayed for their theobromine content by the three methods listed below.

Gravimetric Method of N. F. VIII.—The procedure of the N. F. VIII, page 532, was carefully followed, with particular attention to details.

Method Based on Nitrogen Content.—The Kjeldahl-Gunning-Arnold method with a preliminary digestion on a water bath as proposed by the author (25) was used. This method is as follows: Place an accurately weighed sample of about 350 mg. of theobromine sodium salicylate in a 500-cc. Kjeldahl flask and add 15–18 Gm. of anhydrous sodium sulfate together with 1 Gm. of cupric sulfate and 25 cc. of fuming sulfuric acid. Heat the flask with contents on a boiling water bath for thirty minutes, occasionally shaking it so that the digestion mixture comes in direct contact with all of the sample. Remove the flask from the water bath and place in an inclined position in the usual manner. Heat the mixture gently until frothing ceases, making sure that the temperature is maintained below the boiling point of the acid. Gradually increase the heat until the acid boils briskly, and digest for two hours after the mixture has turned green or until oxidation is complete. After cooling, dilute with about 100 cc. of water and add a few pieces of granulated zinc to prevent bumping. Add sufficient concentrated sodium hydroxide solution to make the reaction strongly alkaline, pouring it down the side of the flask so that it does not immediately mix with the acid solution. Connect the flask to a condenser by the use of a connecting bulb and place the tip of the condenser below the surface of 50.00 cc. of 0.1 *N* sulfuric acid contained in the receiver; carefully mix the contents of the flask by shaking and distill until all of the ammonia has passed into the measured excess of standard acid. Titrate the excess 0.1 *N* sulfuric acid with 0.1 *N* sodium hydroxide solution using methyl red T. S. as the indicator. Each cc. of 0.1 *N* sulfuric acid is equivalent to 1.401 mg. of nitrogen or 4.504 mg. of theobromine.

Suggested Acidimetric Method for Next Revision of N. F.—Transfer an accurately weighed sample of about 1 Gm. of theobromine sodium salicylate, previously dried to constant weight at 110°, to a 500-cc. wide-mouth Erlenmeyer flask. Add 75 cc. of distilled water and 40 cc. of approximately 0.1 *N* sulfuric acid. Boil for two to three minutes, washing down the sides of the flask with three 15-cc. portions of distilled water. Cool quickly to about 40° and add 1 cc. of phenol red T. S. Add a slight excess of 0.1 *N* sodium hydroxide to make the solution alkaline and then carefully adjust the *p*H of the solution by adding the minimal amount of 0.1 *N* sulfuric acid required to produce a lemon-yellow color. Add 35 cc. of 0.1 *N* silver nitrate and titrate the liberated nitric acid slowly with 0.1 *N* sodium hydroxide, to the first sign of a bluish red color. Approach the end point drop by drop. Each cc. of 0.1 *N* sodium hydroxide is equivalent to 18.017 mg. of theobromine.

RESULTS AND DISCUSSION

Table I gives the results on the determination of theobromine in the ten commercial samples by the

three methods. The results are typical of those obtained in these laboratories during fifteen years of experience with the methods outlined above. The tabulated results are averages of at least two, and in some cases three, determinations, all of which were made on the anhydrous sample.

A study of Table I shows that the proposed acidimetric method yields results which are in excellent agreement with the standard method using nitrogen as a basis for calculating theobromine. On the other hand, a similar comparison with the present National Formulary method shows that of the ten samples tested the results average 1.39% higher, on an absolute basis, than by the other two methods. This corresponds to a relative difference of approximately 3%. One can readily see from Table I that all of the samples meet the minimum requirement of 46.5% of theobromine when tested by the official method. However, when tested by the proposed acidimetric and nitrogen methods some of the samples fail to meet the official standards while others would barely pass. In other words, some of the theobromine sodium salicylate being used today would fail to meet the official requirements of the National Formulary if a more accurate method for the determination of the theobromine content were adopted. This again emphasizes the inadequacy of the official method.

In addition to being more accurate, the proposed volumetric method presents several other important practical advantages. It is simple in operation, convenient to use and, most of all, very rapid. A complete theobromine determination can be run by the acidimetric method in a matter of minutes whereas several hours elapse before results can be obtained with the official gravimetric method. Another important practical advantage of the method is its wide applicability in determining theobromine in combination with various other medicaments and also in various pharmaceutical forms such as tablets. The usual tablet diluents and excipients do not interfere.

TABLE I.—DETERMINATION OF THEOBROMINE BY THREE METHODS

No.	Sample	Theobromine Content from Nitrogen, %	Proposed Acidimetric Method: Theobromine Content, %	Proposed Acidimetric Method: Difference, %	Present N. F. Method: Theobromine Content, %	Present N. F. Method: Difference, %
1	Theobrom. et Sod. Salicyl. N. F. VIII "A"	46.67	46.69	+0.02	48.92	+2.25
2	Theobrom. et Sod. Salicyl. N. F. VIII "B"	46.28	46.19	−0.09	47.15	+0.87
3	Theobrom. et Sod. Salicyl. N. F. VIII "C"	47.03	47.14	+0.11	48.81	+1.78
4	Theobrom. et Sod. Salicyl. N. F. VIII "D"	46.51	46.39	−0.12	47.52	+1.01
5	Theobrom. et Sod. Salicyl. N. F. VIII "E"	46.85	46.76	−0.09	48.03	+1.18
6	Theobrom. et Sod. Salicyl. N. F. VIII "F"	46.19	46.25	+0.06	47.70	+1.51
7	Diuretin "A"	46.40	46.25	−0.15	48.04	+1.64
8	Diuretin "B"	46.95	47.05	+0.10	48.33	+1.38
9	Diuretin "C"	46.50	46.49	−0.01	47.61	+1.11
10	Diuretin "D"	46.64	46.70	+0.06	47.80	+1.16
Mean difference		...	...	−0.01	...	+1.39

SUMMARY

1. The literature on methods for the determination of theobromine in Theobromine and Sodium Salicylate is reviewed.

2. The disadvantages of the present National Formulary method are discussed.

3. Ten commercial samples were analyzed for their theobromine content by three different methods: (1) the present National Formulary VIII method, (2) the proposed acidimetric method, and (3) from the nitrogen content as determined by a modification of the Kjeldahl method. The results show that the present official National Formulary method gives high results while the acidimetric method gives results which are in close agreement with those calculated from the nitrogen content.

4. Compared with the official method, the proposed procedure features ease of operation, saving of time, and a high degree of precision and accuracy.

5. The author recommends that a study of the proposed method be made with the view of its adoption as a standard National Formulary procedure.

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Scotch Mint and Spearmint. A Comparative Stud of Cultural, Morphological, and Histological Character istics of Species of *Mentha* Growing in Florida. III*

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FOR OVER a century, the United States Pharmacopœia has recognized as Spearmint only the one species, *Mentha spicata* L. (*M. viridis* L.). Within recent years, however, the commercial crude drug and its volatile oil on the one hand and the current Pharmacopœial description on the other have not always corresponded in all respects to the species named. A "variety" known as "Scotch Spearmint" has become popular with the American growers. As this is apparently a true species, quite distinct from *M. spicata* (the reasons are detailed in this paper), it would seem manifest that recognition of and data for this entity should appear in the official monographs.

STUDIES

The species *Mentha spicata* is well characterized taxonomically, i.e., differences of various specimens in the chief botanical characters are relatively slight. Its inflorescence is made up of verticillasters (pseudo-whorls), comprising a "spike" which is characteristically compact and continuous except close to the base, but occasionally is more or less broken by growth of the internodes separating the "whorls." Always, however, the spiciform habit in this species is pronounced, with no foliage leaves composing part of the inflorescence proper. The glabrous or subglabrous foliage leaves stand below the inflorescence and are definitely sessile, or at the most (such as on lateral axes) with inconspicuous petioles.

The author's interest in the subject was initially attracted by the U. S. P. XII monograph, where petioles up to 12 mm. in length were permitte Up to and including U. S. P. VIII, the leaves we described as subsessile or "usually sessile." U. S. I IX, X, XI, and XIII permit petioles up to 5 mn long. However, the author's studies (describe later) show that *M. spicata* leaves do ordinari exhibit petioles longer than 3 mm., leaves with long stalks originating from a separate species, presun ably *M. cardiaca* Gerard, forming the main sul ject of this paper. This is only one of several signif cant differences in the species, to be noted later.

Entities Having Spearmint Odor.—Several vari ties of *Mentha spicata* L. em. Hudson (*M. spica* (L.) Hudson) have been grown for their herb an volatile oil. Of the 29 varieties of the species liste by John Briquet (1) alone, the following are said t be cultivated to some extent: var. *tennis* (Mx. Briquet, said to be a common U. S. cultigen; va *trichoura* Briq., claimed common in England; va *crispata* (Schrad.) Briq. (*M. crispata* Schrad.), grown in Germany; and other varieties grown to smaller extent in civilized temperate countries.

The penetrating and characteristic aroma an taste of spearmint[3] are possessed in common by number of species of *Mentha*, some of which ar regularly used, others not. At least one plant from a different genus and family also possesses the characteristic odor of spearmint, viz., *Tanacetum balsamita* L. (*Compositae*). Spearmint odor-taste is said to be observed in *M. longifolia* (L. pp.) Hudson

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[1] There has been considerable confusion over the Crisped or Curled Mint of Germany and other Continental countries. While most Folium Menthae Crispae originates from *M. spicata* var. *crispata*, a certain amount also appears to come from *M. longifolia* Huds. var. *undulata* Briq. (2) and from other species, some of which have actually a quite different aroma from that of spearmint. Tschirch (3) has made the rather broad generalization that all crisped mints have a spearmint-like odor. Briquet compiled for him (3) 20 varieties included in 9 species (inclusive of *M. spicata* with 6 such varieties), which have crisped leaves. Flueckiger (4) noted that the German Pharmacopœia II recognizes under *M. crispa* any *Mentha* species with crisped leaves, as long as it has sufficient carvone in its oil. Hence, *M. spicata* varieties with flat uncrisped laminas and *M. Cardiaca* were excluded.

[2] Topitz (5) listed 13 forms of *M. spicata;* others were proposed by Opiz, Lejeune and Courtois, H. Braun, *et al.* In addition, other varieties are occasionally referred to by horticulturists, e.g., "var." *anisatus* ("anise-scented mint"), etc.

[3] Pleasant to most but not all people, and described by some as coarse and unpleasant.

(*M. sylvestris* L.),[4,5] and some varieties, such as *mollissima*;[4] *M. niliaca* Jacq. f. *nemorosa* (Willd.) Briquet (= *M. longifolia* (L.) Hudson var. *nemorosa* ? = *M. nemorosa* Willd. ?);[4] *M. rubra* Hudson (possibly synonymous with *M. Cardiaca*);[4] *M. gentilis* L. (6);[6] *M. aquatica* L. var. *crispa* Benth. (*M. crispa* L.);[8] *M. alopecuroides* Hull (7);[4] *M. verticillata* L. (= *M. thuringiaca* H. Br. & Top. = *M. dalmatica* Tausch f. *thuringiaca* H. Br. & Top.);[6,7] and in *M. cardiaca*.[4] In addition, the following species have sometimes been said to have spearmint properties: *M. javanica* Blume (*M. arvensis* L. var. *javanica* Benth.);[8] a few forms of *M. canadensis* L.;[6] *M. pratensis* Sole;[8] *M. rotundifolia* (L.) Huds.[6] (8); *M. villosa* Huds. (7, 9);[8] and others.[9] As happens not uncommonly, it is likely that the record here is confused by frequent wrong identifications,[10] resulting from careless or hurried work, or the lack of specialized study of adequate reference materials.

The odor of Spearmint (etc.) was frequently stated to resemble that of Basil (*Ocymum* species),[6] hence the use of such synonyms as *M. ocimiodora* Opiz (= *M. spicata* L. per De Candolle (12)).

Spearmint plantings in U. S.—Field studies in Michigan undertaken by the author have confirmed that there are now two types of spearmint grown in that area: (*a*) "Native (American) Spearmint," also known variously as "Common Spearmint," "Ordinary Type," "True Variety" (so called because the original sort used), "American spearmint," etc. This is the well-known *M. spicata*, possibly in more than one variety (Fig. 1). Undoubtedly, this is the first type of spearmint found in America, having apparently been reported in 1672 by John Josselyn and *ca.* 1739 by Gronovius (13) as a naturalized plant, later by others (14, 15). (*b*) Scotch (Scottish?) Spearmint, also sometimes known as "Highland Mint,"[11] etc. A better common name would be Scotch mint, since this would more emphatically indicate its specific distinction from spearmint (Figs. 2, 3).

Fortunately, a brief account of the early history of Scotch mint was obtained from Mr. Richard F. Stroud, of Kalamazoo, Mich. (personal communication, Dec. 10, 1947), who has had an experience of about fifty years in the cultivation of the mints and other medicinal plants. He states:

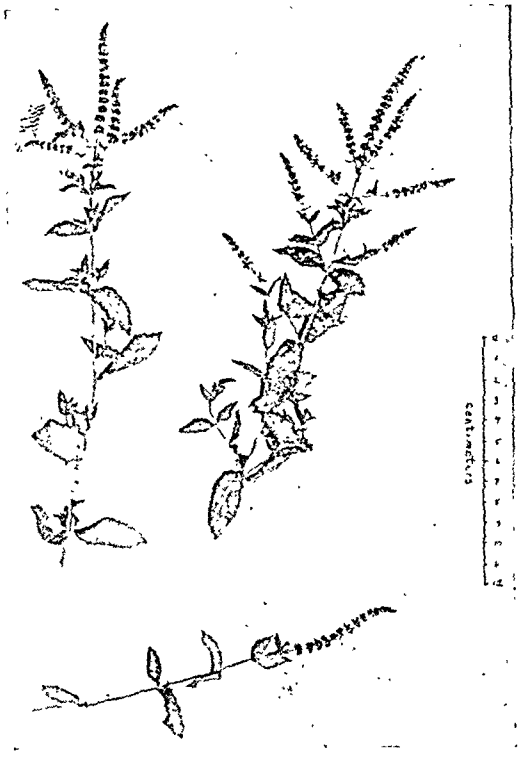

Fig. 1.—*Mentha spicata* L. Specimen from cultivation near Lafayette, Indiana, 1941.

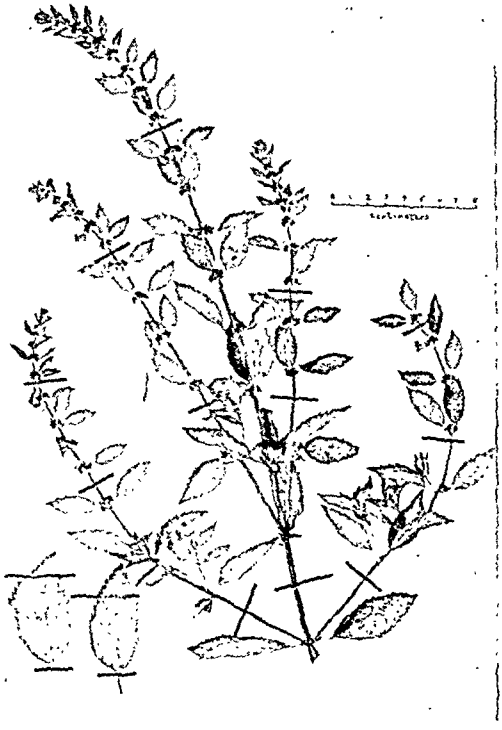

Fig. 2.—Scotch Spearmint. *Mentha cardiaca* Gerard. Specimen collected in Michigan and lent by A. M. Todd Company, Kalamazoo, Mich., 1944.

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[4] Aroma corroborated by the author.

[5] The older English a[illegible] to this species as spearmint. Fc [illegible] l: "The leaues (*Sic!*) of Speare M [illegible] Willow tree but whiter, softer, a[illegible] more [illegible].

[6] Could not be confirmed from available specimens. Negative findings are not however reliable, since some specimens of mint lose their aroma after only a few years, while in other cases the odor is manifest after scores of years of storage, sometimes even after a century!

[7] Var. *strabala* Brig. of this species is said to be widely cultivated in Russia to furnish spearmint type of herb and volatile oil (Russian Crisped Mint).

[8] Specimens not available to writer.

[9] Ruttle (11) indicates there are several different kinds of spearmint, as denoted by varying chromosome numbers. She recognizes that much of the spearmint of commerce is definitely not *M. spicata* but at the most a hybrid of this with other species.

[10] While the characteristic odor of spearmint is a convenient means of readily confirming identification, it may if carelessly used lead to errors in determination of *Mentha* specimens, hence is not a character ordinarily used by taxonomists.

[11] Heard used by workmen at Mentha, Mich. Mr. Stroud says: "The name 'Highland' was given to the plant by me in 1910 when I first had it in the experimental garden at Mentha and I doubt if that name has followed it beyond that vicinity although Dr. Ray Nelson has . . . decided to call it 'Mentha spicata var. Highland!' "

"A year or two previous to 1910 the late Mr. A. E. Beebe, on a vacation up in Wisconsin ran across the plant in a Scotchman's garden. This man had brought it from Scotland for culinary use. Mr. A. M. Todd secured a few roots from Beebe in 1910 and these I extended to several hundred acres in the course of a few years. Roots from these plantations were sent to St. Johns, Mich., and many other points so that now in 1947 I would conservatively estimate fully 75% of all spearmint produced in Michigan and Indiana is of the Scotch variety."

Guenther (16) also mentions the plant's introduction in 1910 into commercial cultivation. Apparently it increased in popularity rather slowly, however.[12] The most conservative current estimate is that about equal acreages are planted to each species (16). Harmer (68) recommends the Scotch "variety" for Michigan muck soils. An outstanding Michigan producer (17) estimates that 75% of the spearmint acreage in both that state and Indiana is composed of Scotch spearmint.

This impression that "true" spearmint is now much less common was also obtained from a field survey by the writer, who found only the Scotch type being grown in the vicinity of Charlotte, Potterville, and Lansing, Mich. Only at Mentha was a considerable acreage of *M. spicata* observed. Studies were made both of living plants in the field and (since it was late in the season) of the charges of exhausted hay (locally called "cheeses") brought from the still into the fields.

An important firm in Indiana (19) reports: "There is no single field of Native . . . Spearmint left any more in . . . States producing this oil. 98% is Scotch-type Spearmint and what little of the native or ordinary there is, same is mixed in the Scotch-type fields." Another important producer says: "With few exceptions, of small plots, Scottish is being raised entirely in Michigan. It produces a much larger yield than the native" (1948). In a study of northern Indiana mint fields, Smith and Robertson (20) said that in 1939, of 11 old settings of rhizomes, 3 were of "regular" spearmint and 8 of "Scotch," and of 9 new settings, 2 were "regular" and 7 "Scotch," showing here a net average of 75% "Scotch type" in the commercial plantings studied.

In the Michigan mint fields studied,[13] it was noted that the "Scotch" mint was set out in rows 3 feet apart with plants about one foot apart in the row, whereas native mint was placed much closer together in the row (about 4 inches between plants). The first year's growth is called "Row Spearmint," the second and later years' "Meadow Spearmint" (a result of the plant's spreading out between rows). As nearly as can be determined, Spearmint is not being produced in any quantity in Washington, Oregon, or other states, at present. Several years ago, one or more experimental plantings of Scotch mint are said to have been laid down in Oregon, but these seem not to have been continued (21).

While Scotch mint and common spearmint are readily separable by their botanical characters, the habit of these species is remarkably similar, a fact which has been noted frequently.[14] Hence, it is not surprising that some of the growers do not know which species they have, nor do the buyers always distinguish between the oils (22).

Distribution of *Mentha cardiaca*.—This species and the closely related *M. gentilis* (and varieties) are reported as cultivated in farm gardens in Central Europe (23), and also occur there wild though never spontaneously.[15] In England, *M. cardiaca* was found in "waste places" but "scarcely wild" (24) and is reported from 5 of the 71 "vice-counties" of Great Britain (25). It is grown in gardens, in Somersetshire, Wiltshire, Kent (26, 27), Surrey (28) and Oxfordshire (26, 29). The species is reported wild or quasi-wild from Yorkshire (30) and Surrey (28), Gower in South Wales (31), Durham, Oxfordshire, near Hertford, Middlesex, Norfolk, Leicestershire (32), and at such times has been referred to as a "denizen looking native but doubtfully indigenous." The species seems to have had a long history of cultivation (which may account for its superior properties), and is occasionally referred to as growing in medieval monastic gardens (28, 31). Fries (33) speaks of cultivation in Sweden and of spontaneous growth in a cultivated field at Lund up to 1823, when it yielded to cereal grains.

Similarly, in our country, *M. cardiaca* does not seem to have escaped widely from cultivation. It is not commonly treated in floras or collecting lists. Britton and Brown (34) report the range from Nova Scotia to New Jersey, Pennsylvania, and the District of Columbia, which can naturally now be extended to the states in which cultivated. Deam (35) notes finding the species growing spontaneously only once (in 1922) in Indiana, a state where many hundreds of acres of the species now receive cultivation. In Michigan, it was not mentioned by Beal in 1904 (36) (as would be expected at that early date), but was reported in 1923 from Fraser, Mich., and near by ("large patch") by Farwell (37), and again by Farwell in 1924 from Monroe Piers (6).

In the Medicinal Plant Garden of the University of Florida, as in many other gardens, this species has been grown as "spearmint," and appears to have originated there from plantings made in or about April, 1926, of what appeared in the records as "Scotch spearmint, *Mentha viridis* L." (Scotch spearmint was mentioned in the records again in 1927 and 1928.) These plants were reported to show good, actually profuse, growth, and to be spreading rapidly, doing especially well where the planting bordered the shade in the moister part of the Garden. Although not specified as "Scotch" in succeeding records, this species was grown in the Garden up to the present time, as proved by preserved herbarium specimens and photographs. It appears that in the course of the years, the "ordinary" variety of spearmint disappeared from the Garden, while the Scotch mint remained as a vigorous growth.

Examination of an herbarium specimen and of photographs of the "spearmint" of the Florida Garden, representative of the material used in the

[12] Thus, this species is not referred to in Bailey's Manual (18), published in 1924, presumably because it was not at that time an important commercial spearmint. The spearmint formerly a commercial crop in New York State was no doubt *M. spicata*.

[13] Visited among others were: Mentha, Mich.; James McCloud, Charlotte, Mich.; Muck Experimental Farm, near Lansing, Mich.

[14] However, it appears to the writer that generally speaking the herb of *M. Cardiaca* plants is a lighter green in color, often with reddish stems, and has strikingly bright pink flowers.

[15] The American vernacular name, Native Spearmint, is a misnomer, since *M. spicata* like most mints was introduced from Europe.

work of Christensen and Hiner (39), proves that this cultigen was *M. cardiaca*. Hence, the data reported in their paper relate not to *M. spicata* but to *M. cardiaca*. The same species was still under cultivation in the Florida Garden during 1940–1945.

NOMENCLATURE

Botanical Synonyms.—There is still some of the confusion in the species *Mentha sativa*, *M. gracilis*, *M. rubra*, and *M. gentilis*, that was manifest over a century ago (40, 41). What gives rise to this "unlimited confusion" (*grenzenlose Verwirrung*) (Ascherson) is in part the considerable tendency to variation of a number of important diagnostic characters, including plant height, leaf form, inflorescence type, form of calyx and calyx lobes (41).

There is no question that *M. cardiaca* is very closely related to the species *M. gentilis*, and Briquet's presentation of it as a variety of this species (71) may accurately reflect its true position. This classification is preferred by many systematists (the so-called "lumpers") who seek to reduce the number of recognized species and thus simplify and coordinate taxonomic names by subordinating many species to the rank of subspecies, variety, and other designations. Descriptions for *M. gentilis* as outlined by a number of writers, such as Small (42), Rydberg (43), Britton (44), *et al.*, appear inclusive of *M. cardiaca*. Both *M. gentilis* and *M. cardiaca*, as well as *M. sativa*, *M. gracilis*, and *M. rubra*, are generally believed to be hybrids of *M. arvensis* L. × < *M. spicata* L. By this means, both the verticillate inflorescence and the frequently appearing spearmint-type odor of these species receive rational explanation.

In addition to the four species named above, *M. cardiaca* is sometimes also confused with other verticillate species, such as *M. arvensis* or *M. canadensis*. For instance, Bentham includes *M. gentilis* as a synonym under *M. arvensis* (40, 12).[16]

Layman confusion with *M. spicata* has already been mentioned, but the plants' similar habit has also sometimes confused serious students.

M. cardiaca Gerard[17] ex Baker
  Synonyms
    M. gentilis L. var. (forma) *cardiaca* (Smith) Briq. (71)
    M. gentilis L. subsp. 3 *cardiaca* Briq. (48)

Other botanical names which have been applied to this entity include:[18]

Sisymbrium sativum Matthiolus (10)
M. hortensis verticillata Ocimi odore Morison (47)
M. gracilis Smith var. β *Cardiaca* Smith (32)
M. cantalica Héribaud (49)
M. gracilis Sole (9)
M. gentilis L. β *glabrata* Fries

Vernacular Names.—The significance of the term "Scotch" as applied to this mint could not be established from the literature. The introduction of the plant from Scotland is referred to in an earlier paragraph and this may account for the name. The synonym was not, however, found in works on the flora of Scotland nor in the various exhaustive works on common names. Although reported apparently for Scotland by Sibbald (1684), Lightfoot (1777), Hooker (1821), *et al.*, the species is believed to have originated on the Continent. Another possible explanation for the common name lies in the occasional definition of "Scotch" as "to make incision in, notch, gash, score" (50). (Cf. "scotches," an agricultural term signifying scores or notches (51).) (Cf. also "scutch.") As noted elsewhere, the "notched" spike of Scotch mint is a ready distinction from the relatively regular "ungashed" spiciform inflorescence of true spearmint. Hence, Scotch mint may possibly have derived from "Scotched Mint." The name "spearmint" as used in association was applied no doubt from the odor and because of the sharpness of the leaves (52), as well, of course, as the resemblance in habit of the plants.

The name "Cardiac Mint" (9) and the specific name *cardiaca* originated most probably from the Latin "cardiacus" = heart- or stomach-affecting (53), because often used for treating "heartburn," gas on stomach and other similar ailments. (Cf. use of after-dinner mints.) This Latin term had as antecedent the Greek καρδία meaning heart, or the upper or cardiac orifice of the stomach, where the esophagus enters it (54). "Cardia" is also an English medical term having this latter meaning.

Other synonyms which have occurred at times are:[19]

Cardiaca (10)
Cardiaca Mentha (10)
Hartwurt (55)
Hart(-)woort (56)
Hart Mint (10, 55, 60); Hart Mynte (55)
Heart-Mint (56)
Her(t)zkraut (in "High Dutch") (10, 56)
Hertzmüntz (57)
Herbe de cueur (55)
Littleleaf Mint (58)[20]
Small-leaved Mint (34)[20]
Narrow-leaved Mint (24)
(True) Garden Mint (Somersetshire, Wiltshire, Kent) (8, 26)
Speare Mint (60)
"Mint" (Oxfordshire) (29)
Red Mint (62)
Red Sharp-leav'd Mint (?) (in Scotland) (Lightfoot)
Bushy Red Mint (?) (in Scotland) (Hooker)
Scotch Variety (or Type) of Spearmint (U. S. growers) (20)
"Highland Mint" (highly localized, Mich.)
Kukenbalsam (in German) (56)
Edelminze (in German) (23)
Edle Münze (in Swiss German) (63)
Edelmüntze (also applied to *M. gentilis*)[19]
Sisembrio Domestico (in Italian) (56)
Balsamita (in Italian) (56)
"Flora Coronata" (?) (55)

[16] To illustrate with one of many other examples, the inadequate description of *M. arvensis* by Wood (46) might well include both *M. gentilis* and *M. cardiaca*.

[17] The spelling as "Gerarde" occurs on the title page of the Herball (10), but although commonly adopted is undoubtedly in error (38).

[18] Acceptance is based on description or, preferably, where available, on herbarium specimens or figures. Many other synonyms which could not be checked have been compiled by various authors, e.g., Sole, Fries, Baker, *et al.*

[19] Some synonyms for *M. gentilis* are also applicable, such as: Gentile Mint; Herba (Menthae) Balsaminae; "Meadow Mint"; etc.

[20] So called because of the characteristic small (floral) leaves subtending the verticillasters.

CHARACTERISTICS

Botanical Characters of Scotch Mint.—*Mentha cardiaca* may be distinguished from other mint species which it resembles (*M. spicata*, *M. arvensis*, *M. canadensis*, *M. gentilis*, etc.) by the following characters (arranged in approximate order of importance): (1) Leaves short petiolate to petiolate throughout (in *M. spicata* sessile or very short petiolate).[21] This character alone is inadequate, since petiolate forms of *M. spicata* have been described (e.g., subsp. *exquisita* Bq. (48)). (2) Inflorescence axillary (subgenus *Verticillatae* L. = *Verticillastreae* Mvd. = *Axillares* Roch.), i.e., without the more or less continuous spiciform state seen in *M. spicata*, etc. (subgenus *Spicatae* L. = *Spicastreae* Bq.), or the capitate formation of *M. piperita*, etc. (subgenus *Capitatae* L.). (3) Axillary verticillasters are subtended by floral leaves (distinct from bracts[22]) from the top to the bottom of the inflorescence.[23] These floral leaves are however considerably smaller than the lower cauline foliage leaves and are not of the relatively large dimensions seen in *M. arvensis* and *M. gentilis*, in which there is only slight reduction in leaf size as one proceeds up the stem and passes from the vegetative to the flowering part of the axis.[24] In *M. cardiaca*, the reduced leaves extend down the axis for a considerable distance; in fact, the largest leaf found (subtending a lowermost verticillaster) measured only 35 mm. in total length. The lower verticillasters are rather widely separated. (4) The base of the calyx and the entire pedicel are glabrous and not pubescent as in *M. arvensis*, *M. canadensis*, etc.[21] (Fig. 3, *H*). (5) Calyx and corolla are both glabrous internally. (6) Leaves and stems of *M. spicata* and of *M. gentilis* sometimes are glabrous or nearly so, whereas for *M. cardiaca* these organs are typically quite hairy, but not as densely clothed as generally in *M. arvensis*, etc. (7) Foliage leaves are typically broader, less oblong than in *M. spicata*, but are not as broad as usual for *M. gentilis*, *M. arvensis*, etc.; and are much less obtuse than in *M. gentilis*. (8) Foliage leaves are strongly and sharply serrate, somewhat similar to those of *M. gentilis*, but radically different from those of *M. spicata* (Fig. 3, *B*, *P*). (9) Calyx tubular-campanulate (Fig. 3, *G*, *H*), not campanulate as in *M. gentilis* and *M. spicata* nor tubular as in *M. arvensis*. (10) *M. cardiaca* grows commonly 1.2 to 1.4 m. high and hence is taller than *M. spicata*, at least in cultivation. (11) Foliage is colored a light and bright shade of green. Leaves of *M. gentilis* are characteristically much darker, often distinctively marked on the upper side by whitish areas adjacent to midrib and veins.[25] (12) Twelve to thirteen veins traverse the calyx of *M. cardiaca* (10 in *M. arvensis*, *M. gentilis*). (13) The flower in *M. cardiaca* is decidedly larger than in *M. spicata*, *M. gentilis*, etc., and approaches the flower size in *M. arvensis*. (14) The calyx lobes of *M. cardiaca* are relatively much shorter than the tube as compared with *M. spicata* (Fig. 3, *H*).

Petioles.—*Mentha spicata* is commonly stated to have sessile to subsessile leaves. Topitz (5) describes the leaves of this species and varieties as sessile, subsessile, short petiolate, and subpetiolate, although these last three terms would seem almost identical. Fassett (66) and Sun (67) speak of petioles 0 to 2 mm. in length. Baker (64), the post-Linnaean author of the species, and some other writers, have described the leaves of *M. Cardiaca* as sessile or with the lowest very short-stalked, whereas the writer's observations indicated leaves with medium long petioles throughout. The figure of Gerard (56), who was cited by Baker as the author, indicates definite petioling, and his description speaks of "long leaues . . . in [on] stalks."

To check these points, a study was made of hundreds of herbarium specimens of these two species. Following a careful check on the identity of each specimen, the petioles were examined[26] and a single maximum length was recorded (to the nearest millimeter) for each specimen[27] (Table I).

TABLE I.—DISTRIBUTION OF PETIOLE LENGTHS

	Length, Mm.	No Specimens
Mentha spicata Av. = 1.1 mm.	0	85
	1	112
	2	94
	3	16
	Total	307
Mentha cardiaca Av. = 6.4 mm.	2	3
	3	8
	4	16
	5	12
	6	20
	7	20
	8	16
	9	8
	10	9
	11	4
	12	2
	14	2
	Total	120

[21] Malinvaud recognized characters (1, 4) as "precise and constant" for *M. gentilis* (under which he recognized Cardiac Mint as a variety)

[22] Typical bracts differ from these floral leaves in their scale-like conformation: narrow, elongate, of dark reddish color, provided with two lateral lobes similar in appearance and only slightly smaller.

[23] In *M. spicata*, the lowest verticillaster alone is sometimes subtended by a pair of leaves in the fashion of *M. cardiaca*.

[24] In *M. gentilis*, however, considerable reduction in floral leaf size occurs usually within 1 to 3 cm. of the stem tip There is sufficient variation on this point, however, to make it easy to confuse the species unless care is taken. Thus, in Britton and Brown (34), the genus diagnosis properly separates *M. cardiaca* from *M. gentilis*, etc., but under the spe- [illegible] *M. gentilis* is contradictorily stated to have [illegible] much smaller than the lower," in [illegible] distinction in part. In *M. gentilis* [illegible] ried in the axils of very large leaves (sometimes the largest on the plant, along the center of stem) and down the stem for considerable distances (sometimes over 30 cm.). (In Baker's plate of *M. cardiaca* (64), some "whorls, such as the eighth down, are in the axils of rather larger leaves than found in the writer's observations.) In atypical specimens of *M. gentilis* (hybrids or varieties) where the verticillasters were subtended above by small, below by large leaves, it was found that the floral leaves have longer petioles than in *M. cardiaca*, hence extend farther out. There appears to be a fairly well-defined gradation in size of floral leaves, starting with *M. spicata*, which has diminutive leaves bracketing the lowermost verticillasters Then come *M. cardiaca*, with pronounced leaves, and increasing in prominence, *M. gentilis*, then *M. arvensis*.

[25] This species is said to show an extreme variegated form known as "Golden Apple Mint" (11).

[26] Petiolar length varies within limits according to the amount of moisture available to the plant (61, 65). Although considerable variability in petiolar length is manifest within these species, yet each is still distinctive with little overlapping of limits.

[27] It has been said that leaves taken from the main stem and not from the branches are most typical and should alone be considered. As far as petiolar length, this could not be confirmed. Also, up to about the center of the stem, no recession in petiolar length could be noted as compared with the lowermost leaves.

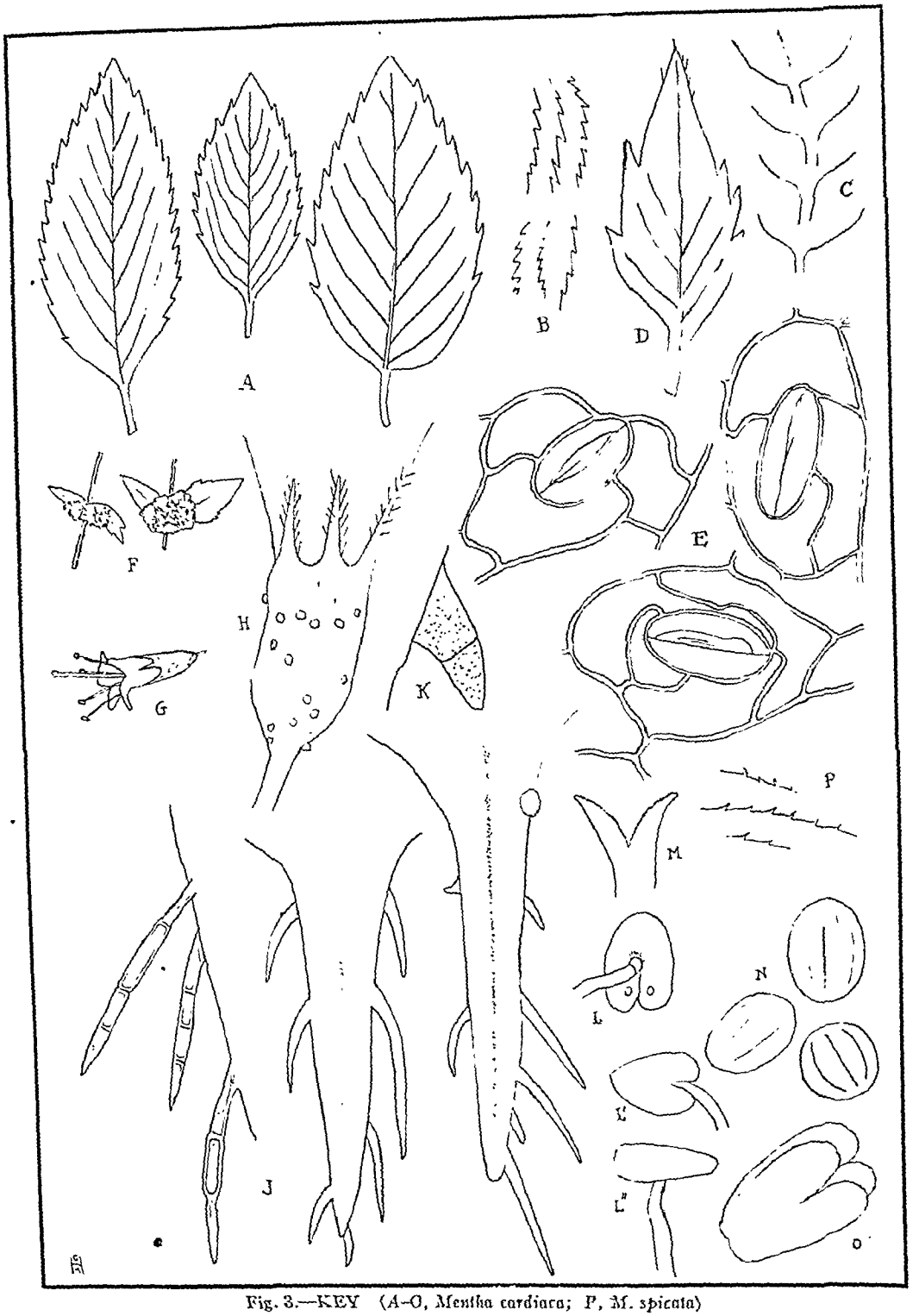

Fig. 3.—KEY (*A–O*, *Mentha cardiaca*; *P*, *M. spicata*)

A, tracings of 3 foliage leaves; *B*, margins of foliage leaves; *C*, bases of foliage leaves; *D*, floral leaf; *E*, stomata from stem epidermis; *F*, verticillasters; *G*, flower with exerted stamens and pistil; *H*, calyx; *J*, calyx lobes with uniserial mechanical hairs; *K*, conical hair on calyx; *L*, stamen from below; *L'*, stamen from above; *L''*, stamen from side; *M*, apex of pistil; *N*, pollen grains; *O*, fruit; *P*, *M. spicata*, margin of foliage leaf.

Enlarged: *D*, *G*, *H*, *L*, *L'*, *L''*, *O*. Much enlarged: *E*, *J*, *K*, *M*, *N*. Others: approx. natural size.

It is clear that leaves of *M. spicata* are sessile or practically so, with petioles occasionally up to 3 mm. long, hence at most short petiolate. In *M. cardiaca*, petioles mostly run between 4 and 8 mm. long, hence its leaves have medium length petioles. The present Pharmacopœial allowance (less than 5 mm.) is then too high for *M. spicata*, but too low for *M. cardiaca*.

Description of *Mentha cardiaca*.—It was thought desirable to present a detailed description of the plant as now cultivated in the Middle West. The descriptions presently available are either too condensed or are at variance to a certain extent with the American plant, possibly because European botanists have distinguished several varieties or forms of the species (such as var. 1, 2, of Baker; var. α, β Smith; forma *gratiosa* Topitz, f. *geniata* Topitz, f. *Sagorskii* Bq.).

The data which follow were compiled from the careful study of scores of specimens, both cultivated and wild-growing (Figs. 2, 3).

Plant 9 to 10 to 14 dm. high.—*Stem* erect, sturdy, with many ascending and spreading branches toward the top; retrorse-pubescent, most marked on the vegetative parts of stem, near nodes, and on angles of stem; often glabrous toward base, somewhat scabrous; frequently reddish, deepening toward base. Internodes 2.6–*4*–5.7 cm. long below inflorescence. *Leaves* petioled, the upper (floral leaves) short-petiolate (2–4 mm.), the lower (foliage) leaves with distinct petioles 2–*6.4*–14 mm. long. Serration of margin sharper, more regular, closer, and coarser than in *M. spicata*, the teeth pointed forward, with the exterior side nearly straight, the interior side usually curved, each measuring 4–*5*–6 mm. x 1–*1.4*–1.8 mm.; sometimes doubly serrate. Shape of lamina lanceolate to oblong-lanceolate, to ovate to ovate-lanceolate to elliptical; leaf lamina relatively elongate typically (length/breadth ratio: 2.05–*2.29*–2.72). Apex acute to acuminate; base usually narrowed, but in larger leaves often rounded or even obtuse, entire (Fig. 3, *C*). Floral leaves much reduced, 2 to 3 times or more exceeding axillary floral clusters (Fig. 3, *D*). Leaf upper surface bright green (sometimes reddish on midrib, veins, and near margin), lower surface lighter. Upper surface generally glabrous, or occasionally hairs on midrib; lower surface pubescent, with scattered hairs mostly along veins and margin, especially toward base. Foliage leaves 35–*57*–88 mm. long (incl. petiole), 16–*25*–37 mm. broad, with 5 to 6 pairs of prominent lateral veins. Floral leaves vary in length 4–*20*–35 mm. (incl. petioles), and 2–*9*–18 mm. in breadth (typical size of leaf 15 mm. from stem apex, 5 x 13 mm.). Petioles densely pubescent. Inflorescence verticillate (*status axillaris* with leaves in *status subbracteosus*), with main axis 7–*12*–30 cm. long, with the greatest concentration of flowers in the upper 8 cm. approx., consisting of a series of 6–*13*–19 mostly well-separated subglobose verticillasters (pseudo-whorls) on the main axis, together with smaller series on lateral branches. Verticillasters are subtended in axils of floral leaves, and separated by from 3–*25*–42 mm.; internode length increases toward the lower part of the inflorescence. A few uppermost axils on stem and branches are mostly without flowers. The succession of verticillasters is ordinarily terminated below at the first pair of floral leaves standing above the origin of the first pair of flowering branches. These lateral branches never exceed the main axis. Verticillasters relatively stout, diam. 5–*11*–14 mm., height 6–8 mm. (Fig. 3, *F*). Diameter of verticillaster to tips of floral leaves 8–*29*–50 mm.; ratio (length floral leaf/length flower) ranges from 2 (upper) to 5 (lower). Fifteen to twenty flowers per peduncle, or 30 to 40 per verticillaster. Bracteoles 2 per peduncle, subulate-linear, short petiolate, lightly ciliate on midrib and margins, otherwise glabrous; 3.7–*4.6*–5.2 mm. long, 0.2–0.6 mm. wide, considerably exceeding flower. Peduncles 1.2–*1.7*–2.7 mm. long, however much elongate in lower flower clusters, where found up to 6 mm. long, glabrous. Pedicels 0.6–0.7 mm., reddish, glabrous, with occasional glands. *Calyx* tubular-campanulate to campanulate; glabrous inside and out except for teeth, which are hispid, also have unicellular glandular hairs; exterior of calyx thickly glandular, glands mostly localized along both sides of the five most prominent ribs; 12–13-veined; in flower, calyx measures 2.0–*2.4*–2.7 mm. long, 0.8 mm. in diameter; persistent in fruit; g een before, brownish red after anthesis. Tube slightly furrowed, 1.3–*1.6*–1.8 mm. long. Teeth linear-subulate, some slightly larger than others, 0.7–*1.0*–1.3 mm. long, hence decidedly shorter than tube. *Corolla* 4-lobed, the broadest upper lobe notched, lobes obtuse; pinkish-lavender, sometimes light pink; 3.7–*4.2*–4.4 mm. long; 2.4 mm. max. diameter; corolla tube exceeds calyx; glabrous inside and out; glandular. *Stamens* included and exserted on same plant; heterostyly noted (in some flowers, exserted stamens with included pistil, in others the reverse; occasionally observed exserted pistil with 2 exserted and 2 included stamens); anthers dehisce introrsely, measure 0.35 x 0.50 mm. (av.). Pollen ovoid or subspherical, grooved, 25–28 mμ av. length, sometimes in 3's (1 large, 2 small). *Pistil* 6 mm. long, typically exserted; style 0.45 mm. long, 0.12 mm. thick (av.); stigmata reflexed. *Nutlets* ovoid, dark brown, 0.7 mm. long, warty, bristly at apex. *Palisade Ratio:* 6.5.[28] *Vein Islet Count:* 13.

Other Characteristics of Scotch Mint.—Comparative tests made by crushing the fresh herb or rubbing the dried herb in the palm reveal little qualitative difference in odor of *M. spicata* and *M. cardiaca*, although it is generally obvious that the latter species is much richer in aroma. Guenther (16) says some customers prefer oil from "common," others oil from "Scotch" spearmint. A leading producer in Indiana states that Scotch mint has a 20% higher oil yield and has more of the "minty" aroma, at the same time with less of the "biting" (pungent) taste. Oil from mixed fields is sold to buyers preferring the more pungent article. The "much higher" oil content of Scotch mint is widely confirmed (e.g., by outstanding Michigan producers), and some report up to 50% increased oil yield from this species. This is naturally an important reason for its popularity with mint growers.

Various operators say the chief disadvantage of Scotch mint is its lesser hardiness to cold weather, its lower resistance to insect and fungal attack (e.g., it is rather vulnerable to the *Verticillium* wilt which has caused such havoc in mid-western mint fields), and its need of being more frequently replanted.

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[28] Cf. figures for *M. spicata:* 6.0–*6.2*–6.8 (59).

Countering these are such stated advantages as Scotch mint's earlier maturity, larger size, bigger yields, and apparent better thriftiness in warmer climates (such as in Florida).

As far as the writer is aware, no comparative studies have yet been made on the oils of *M. spicata* and *M. cardiaca*. Data in the literature for American "spearmint oil" may be based on either species or on both in admixture.

It would seem warranted to resurvey standards for spearmint oil on the basis of botanical origins. Analyses which have appeared in recent years (39, 69, 70) show a far wider latitude of constants and of carvone percentage than required in the official monograph for the oil. A systematic study of the oils is definitely indicated and might reasonably be expected to show a quite distinctive range of values for each species. Such a study is to be undertaken by the author.

The shifting standards for spearmint oil in the U. S. P. over the last sixty-five years are indicated in Table II. Note especially the alterations in optical rotation and the increase in carvone percentage between 1905 and 1936. From what has been said as to its increasing popularity with producers, it would be a good surmise from these data that Scotch mint oil has probably a higher levo-rotation and carvone content than true spearmint oil.

It is therefore recommended that the official monographs for both the herb and the oil be amended so as to include the additional species "*Mentha cardiaca* Gerard ex Baker," the synonym "Scotch mint," and changes as follows in "Description" (of Herb) (additions are set within quotation marks):

Leaves . . . ovate-lanceolate "to ovate, oblong-lanceolate, elliptic" . . . 3 to "9 cm." in length, margin "sharply" serrate. Surfaces "glabrous (*M. spicata*) or" under surface with a few hairs on the veins "(*M. cardiaca*)," nearly sessile "(*M. spicata*)" or "with petiole less than 15 mm. in length (*M. cardiaca*)." Stems . . . green "but generally dusky red or purplish." Verticillasters . . . "spiciform (*M. spicata*) or in separated axillary clusters, each subtended by a pair of floral leaves resembling the foliage leaves in appearance but smaller (*M. cardiaca*); the spiciform inflorescence 3 to 9 mm. (*M. spicata*) or 5 to 14 mm. (*M. cardiaca*) in diameter, and in fruit up to 10 cm. (*M. spicata*) or 15 cm. (*M. cardiaca*) in length (occasionally up to 30 cm. in latter)." Calyx campanulate "(*M. spicata*) to campanulate-tubular (*M. cardiaca*), subequally" 5-toothed. . . "glabrous or nearly so throughout (*M spicata*) or glabrous below with hispid teeth (*M. cardiaca*)," green "(*M. spicata*)" to purplish red "(*M. cardiaca*)." Corolla . . . orange "to pinkish to lilac and purple . . . " its tube shorter than the calyx "(*M. spicata*) or longer (*M. cardiaca*)." Style . . . "exserted." Nutlets . . . "minutely grained (*M. spicata*), warty (*M. cardiaca*), dark brown."

TABLE II.—OFFICIAL STANDARDS FOR SPEARMINT OIL OF THE UNITED STATES PHARMACOPŒIA, 1880–1947

Revision (Publication Date)	d_{25}	$(\alpha)_{25}$	n_{25}	Carvone, %
1880[a] (1882)	0.900 approx.	...	...	...
1890 (1894)	0.930–0.940	...	...	...
VIII (1905)	0.914–0.934	−35—48°	...	...
IX (1916)	0.917–0.934	−38—55°	...	NLT 43
X (1926)	0.917–0.934	−38—56°	1.4820–1.4900	NLT 43
XI (1936)	0.917–0.934	−48—59°	1.4820–1.4900	NLT 50
XII (1942)	0.917–0.934	−48—59°	1.4820–1.4900	NLT 50
XIII (1947)	0.917–0.934	−48—59°	1.4840–1.4910	NLT 50

[a] No standards at all given in U. S. P. 1870 and previous revisions.

Uses of Scotch Mint.—*Mentha cardiaca* has been used in the same manner as true spearmint since apparently as early as the time of Parkinson (1640) (60) or possibly still earlier. The old herbalists considered the plant superior to spearmint as a cardiac ("heart" remedy, really heartburn remedy)[29] and stomachic, but inferior as a febrifuge. Jackson (27) experimented with this species in mint sauce and decided it had a poorer flavor than *M. spicata*.

DISCUSSION

It has been plainly shown that Scotch mint has been and is now an important source of spearmint oil. It also constitutes a portion of the spearmint herb of commerce, since a study of commercial lots of "spearmint herb" by the author showed that Scotch mint occurs very often, both alone and in admixture with *M. spicata*.

[29] The herb has, of course, no authentic reputation as a heart stimulant, as presumed by some (59).

SUMMARY

1. A study has been made of wild-growing and cultivated spearmints of the United States, past and present, in the field as well as from herbarium specimens and textual descriptions, and it has been shown that, in addition to true spearmint, an increasingly large proportion of the commercial herb and oil derives from "Scotch spearmint."

2. Wrongly called a "form" of spearmint, "Scotch mint" is a distinct entity which belongs neither to the species *Mentha spicata* L. nor even to the respective subgenus *Spicatae* L., but to a

different species and subgenus of *Mentha*, viz., *M. cardiaca* Gerard ex Baker of subgenus *Verticillatae* L.

3. A fairly complete description of *M. cardiaca*, and several means of distinguishing it from *M. spicata* (True Spearmint) and other *Mentha* species, are presented.

4. The reported variation of spearmint oil in constitution and assay may be explained perhaps by differences between Scotch mint oil and that of true spearmint, on which latter the earliest standards were based. Studies on the respective oils of the two species are to be undertaken.

5. On the basis of a study of many authenticated specimens of both "spearmints," suggestions are advanced for bringing the official monographs up to date.

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Chemical Composition of Gum Turpentines of Pines: A Report on *Pinus strobus, P. cembra, P. taeda, P. radiata,* and *P. virginiana**

By N. T. MIROV, T. H. WANG,† and A. J. HAAGEN-SMIT†

The physical and chemical properties of turpentine obtained from five species of pines have been determined and are reported in this article.

SOME THREE years ago, in cooperation with the California Institute of Technology, the California Forest and Range Experiment Station began investigating the chemical properties of turpentines from different species of the genus *Pinus*. The primary interest of the Experiment Station in this project was to study the nature of the inheritance of biochemical characters of the genus *Pinus* to supplement other genetic research of the Institute of Forest Genetics. Reasons for selecting turpentines for this purpose and application of the information obtained to the problems of forest genetics are fully discussed in a recent article by one of the authors (1). The interest of the California Institute of Technology—more specifically of the laboratory of bio-organic chemistry—in this project is that of exploring the little-known field of chemical composition of turpentines of the genus *Pinus*.

The results reported in this paper deal with turpentine composition of five species of pines: *Pinus strobus, P. cembra, P. taeda, P. radiata,* and *P. virginiana*. Oleoresin samples used in this project were either obtained by members of the Institute of Forest Genetics or received through the courtesy of different persons as indicated in the text. Turpentine was distilled from the oleoresin at the Institute of Forest Genetics Laboratory, Placerville, Calif., with the assistance of E. F. Kimbrough, and physical characteristics of the turpentine were determined and a trial fractionation was made. The main fractionation and identification of the components were done at the Department of Bio-organic Chemistry, California Institute of Technology at Pasadena, Calif.

This article is the third one written jointly by California Institute of Technology and California Forest and Range Experiment Station. More articles are to follow.

PINUS STROBUS

Pinus strobus (L.) is commonly known as eastern white pine. Its range extends from the White Bay region of Newfoundland to northern Ontario, to southern Manitoba, southward through northern and eastern Minnesota, to Iowa, and northern Illinois and Michigan. Once it was abundant as far south (along the Allegheny Mountains) as northern Georgia. Its variety *Chiapensis* occurs in the mountains of southern Mexico (States of Chiapas, Oaxaca, and Vera Cruz).

Oleoresin for the present analysis was collected by staff members of Lake States Forest Experiment Station. The oleoresin was collected in the northern part of Minnesota (Chippewa National Forest) from fourteen 100-year-old trees about 20 inches in diameter. Altogether 3800 Gm. of oleoresin was received.

The rosin acids did not crystallize as readily as in oleoresin of the yellow pines. On the contrary, a honey-like consistency was retained in storage for a long time. It should be noted that oleoresins of all white, or Haploxylon, pines have this tendency to remain liquid for a long time, while oleoresins of the majority of Diploxylon pines usually become sugary and hard very soon after collecting. Apparently there is a definite difference in the composition of the rosin between the Haploxylon and the Diploxylon groups of the genus. Only a few species of pines have been investigated as to the compositions of their rosins. Lombard (2) reported that oleoresin of white (Haploxylon or soft) pines is rich in abietic acid; oleoresin of hard (Diploxylon) pines rarely contains it, but is characterized by the presence of lævo-pimaric acid.

For the present analysis, the turpentine was distilled from the oleoresin under reduced pressure; toward the end of the distillation the temperature inside the flask reached 205°, and the pressure was reduced to 0.05 mm. The turpentine obtained amounted to 25% of the oleoresin.

The turpentine had the following characteristics:

Density, d_4^{21}	0.8611
Index of refraction, $n_D^{20.6}$	1.4714
Specific rotation, $[\alpha]_D^{21}$	−0.75°

The data obtained in fractional distillations are assembled in Table I. Analytical data are given in Table II.

The main part of the oil consists of *dl*-α-pinene (75%) and β-pinene (15%) as shown by the preparations of nitrosochloride (m. p. 106–107°) and nopinic acid (m. p. 125°), respectively. This is in harmony with the physical constants given in Table I and the

* Received May 15, 1949, from the California Forest and Range Experiment Station, which is maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.

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analytical data in Table II. Tests for Δ^3-carene were negative. The higher boiling fractions, 12–15 of the oil contain, as seen from Table II, terpene alcohols, terpene ketones, mixed with terpene hydrocarbons.

Fraction 16 consists solely of a sesquiterpene hydrocarbon (0.3%). After distillation over Na the physical constants of the fraction were: d_4^{25}, 0.9131; n_D^{25}, 1.4913; boiling point 110–115° at 27 mm. Molecular refraction for this hydrocarbon ($C_{15}H_{24}$) was found to be 64.83; calculated for a sesquiterpene ($C_{15}H_{24}$) with one double bond, it would be 64.40. This points to the presence of a tricyclic sesquiterpene.

Tchermark of the University of Vienna. A sample amounting to 1571 Gm. was collected during the summer of 1947 from 30 trees in "der Forstverwaltung Paal bei Murau, Obersteiermark, Austria," at an elevation of 5400 to 5600 feet. The oleoresin was rather liquid but with some rosin acids crystallized out.

From 1571 Gm. of the oleoresin, 320 Gm. (or 20.4%) of turpentine was obtained. The turpentine had the following properties:

Density, d_4^{26}	0.8522
Index of refraction, $n_D^{27.5}$	1.4654
Specific rotation, $[\alpha]_D$	+13.83°

TABLE I.—FRACTIONAL DISTILLATION OF 550 GM. OF *Pinus strobus*

Fraction	Distillation Range: Pressure, Mm.	Distillation Range: Temperature, ° C.	Distillate, %	Density, d_4^{24}	Index of refraction, n_D^{24}	Specific rotation $[\alpha]_D^{25}$
1	740	154–155	1.0	....	....	.....
2	740	155–156	53.6	0.8590	1.4667	+1.85°
3	740	156–157	18.7	....	....	.....
4	740	157–158	1.3	....	....	.....
5	740	158–159	1.5	....	....	.....
6	740	159–160	2.4	....	....	.....
7	740	160–161	5.4	0.8610	1.4707	−10.23°
8	740	161–162	4.3	....	....	.....
9	740	162–164	2.9	0.8590	1.4738	−6.93°
10	740	164–165	1.6	0.8534	1.4740	−2.89°
11	740	165–170	0.8	0.8611	1.4812	.....
12	1.0	60– 70	0.5	0.8732	1.4840	.....
13	1.0	70– 80	0.6	0.9121	1.4884	.....
14	1.0	80– 90	0.2	0.9281	1.4890	.....
15	1.0	90– 94	0.8	0.9223	1.4915	.....
16	1.0	94–100	0.3	0.9264	1.4974	.....
Residue and loss	...	.....	3.7	....	....	.....

TABLE II.—ANALYTICAL DATA OF FRACTIONS[a]

Fraction	Analysis: Carbon, %	Analysis: Hydrogen, %	Determined by Zerewitinoff[b] Method: Active H	Determined by Zerewitinoff[b] Method: Carbonyl Group
3	87.94	12.28	.....	.....
6	88.35	11.79	.....	.....
12	87.30	11.81	0	0.2 mol.
15	84.24	11.57	0.2 mol.	0.4 mol.
16	88.06	12.11	.....	.....

[a] Calculated: for $C_{10}H_{16}$ or $C_{15}H_{24}$: C = 88.17%; H = 11.83%; for $C_{10}H_{16}O$: C = 78.91%; H = 10.58%.
[b] Soltys, Arnulf, "Die micro analytische Bestimmung der aktiven Wasserstoffes nach L. Tschugaeff und Th. Zerewitinoff," *Mikrochemie*, 20, 107(1936).

PINUS CEMBRA

Swiss stone pine (*Pinus cembra*) is a European pine growing at high altitudes in the Alps, from Savoy to the Carpathians. Some botanists maintain that *P. sibirica* of the vast plains and mountains of northeastern Russia and of Siberia is botanically identical with *P. cembra*, and that scrubby *P. pumila* of northeastern Siberia and northern Japan is a variety of *P. cembra*. *Pinus pumila* turpentine has never been analyzed, but that obtained from *P. sibirica* was partly analyzed by Schkateloff (3). A sample of old, oxidized oleoresin yielded only 6% of turpentine which was found to be dextrorotatory, $[\alpha]_D = +14°$, having density, $d_4^{15} = 0.865$; 20% of it boiled between 155° and 156°.

Oleoresin for the present analysis was obtained through the assistance of Professor Dr. H. Knüchel, of Zurich, Switzerland, and Professor Dr. Leo

The fractionation was carried out at ordinary pressure in the usual manner. The fractionation data and physical constants are given in Table III.

From fraction 3 a nitrosochloride was prepared; its melting point was 106–107°, identical with that of α-pinene. After oxidation of fraction 10 with $KMnO_4$, nopinic acid was isolated with a melting point of 126–127°, identical with that obtained from β-pinene. Tests for dipentene on fraction 13 were negative since no tetrabromide could be separated. Tests for Δ^3-carene were negative. The main constituents of the oil are, therefore, α-pinene, 85%, and β-pinene, 9%.

The amounts of higher-boiling constituents show a small maximum, probably in the boiling range173–175°, at 742 mm. This fraction contains mostly mixtures of isomerization and oxidation products of pinene from which no crystalline derivative could be prepared.

Fraction 15 dehydrogenated with sulfur did not give cadalene or other naphthalene hydrocarbon.

PINUS TAEDA

Loblolly pine (*Pinus taeda*) occurs in the southeastern United States from New Jersey to central Florida; west to eastern Texas, northward into southwestern Oklahoma, southwestern Arkansas, and the southern border of middle and west Tennessee.

Loblolly pine turpentine had been previously obtained by Charles H. Herty. His sample was dextrorotatory (+46°); its density, 0.8525, and

index of refraction, 1.4700; fractionation yielded the following results:

9.3% came over between 160–165°
38.0% came over between 165–167°
37.5% came over between 167–172°
12.5% came over between 172–180°
2.7% residue boiled above 180°

From these results it was concluded that the volatile oil from loblolly pine is a mixture of pinene and limonene, with the latter probably in excess. No identification of the components was made.

Oleoresin of loblolly pine used in the present work was sent by Mr. Keith Dorman, of Southeastern Forest Experiment Station, Lake City, Fla. Upon distillation under 0.05 mm., 3818 Gm. of oleoresin yielded 710 Gm. or 18.6% of turpentine. The relatively low yield may be explained by the loss of some volatile oil from a jar broken in transit.

The turpentine had the following constants:

d_4^{22}	0.8570
Index of refraction, $n_D^{27.5}$	1.4675
Specific rotation, $[\alpha]_D$	+20.17°

A sample of loblolly pine turpentine oil was first distilled into three fractions—the low, medium, and high boiling—and each of these fractions was further fractionated. The combined results of these fractionations and the physical properties of the fractions are given in Table IV.

The data in Table IV show that the oil consists largely of *d*-α-pinene, 85%, and *l*-β-pinene, 12%. The nitrosochloride of fraction 5 melts at 106–107°, and gives no depression when mixed with an authentic sample of *d*-α-pinene nitrosochloride. The physical properties of fractions 8 and 9 indicate the presence of *l*-β-pinene. A sample of fraction 9 was oxidized with an aqueous solution of potassium permanganate and sodium hydroxide, and nopinic acid was obtained. The acid after recrystallization melted at 125–125.5°, while the melting point of nopinic acid as recorded in literature is 125°.

The boiling point range, 168–172°, of fractions 11 and 12 suggested that Δ^3-carene might be present, but attempts to prepare Δ^3-carene nitrosate from these fractions failed.

While fraction 13 gave carbon hydrogen values showing the presence of $C_{10}H_{16}$ hydrocarbon, tests for limonene were negative. In a test with fuming sulfuric acid no saturated hydrocarbons could be isolated.

PINUS RADIATA

Pinus radiata (D. Don) is the Monterey pine of California. It grows naturally in a few places on the

TABLE III.—FRACTIONAL DISTILLATION OF THE VOLATILE OIL FROM *Pinus cembra*

Fraction	Distillation Range: Pressure, Mm.	Distillation Range: Temperature, ° C.	Distillate, %	Density, d_4^{20}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	742	100–154	0.8	....	....	.....
2	742	154–155	25.8	....	....	.....
3	742	155–156	45.0	0.8539	1.4640	+18.38°
4	742	156–157	6.0	0.8540	1.4639	+17.63°
5	742	157–158	3.5	....	....	.....
6	742	158–159	3.5	....	....	.....
7	742	159–160	1.0	0.8557	1.4668	+11.10°
8	742	160–162	1.0	....	1.4706	+10.34°
9	742	162–164	2.5	0.8580	1.4690	+ 7.75°
10	742	164–166	1.5	....	....	.....
11	742	166–168	2.5	0.8575	1.4744	+ 2.92°
12	742	168–173	0.5	....	....	.....
13	742	173–175	1.3	0.8684	1.4790	+ 3.10°
14	742	175–180	0.5	....	....	.....
15	742	.....	3.4	....	....	.....
Residue	...	.....	0.2	....	....	.....

TABLE IV.—FRACTIONAL DISTILLATION OF THE VOLATILE OIL FROM *Pinus taeda*.

Fraction	Distillation Range: Pressure, Mm.	Distillation Range: Temperature, ° C.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	740	100–144	0.2	0.8626	1.4576	.....
2	740	144–152	0.40	0.8556	1.4639	.....
3	740	152–154	1.60	0.8546	1.4645	+33.0°
4	740	154–156	16.50	0.8548	1.4650	+31.67°
5	740	156–158	41.60	0.8548	1.4669	+23.74°
6	740	158–160	25.20	0.8555	1.4674	+15.56°
7	740	160–162	1.90	0.8556	1.4691	+12.36°
8	740	162–164	3.8	0.8592	1.4725	− 3.38°
9	740	164–166	2.6	0.8608	1.4743	− 5.52°
10	740	166–168	1.2	0.8605	1.4822	.....
11	740	168–170	1.2	0.8686	1.4790	.....
12	740	170–176	1.7	0.8686	1.4750	.....
13	740	176–196	1.0	0.8570	1.4750	.....
14	740	196–208	0.5	0.9455	1.5016	.....
Residue	...	.....	0.6	....	....	.....

coast south of San Francisco, particularly on the Monterey peninsula and some near-by islands. It has been planted very extensively all over the warmer parts of the world, and in Australia, where it is known as *Pinus insignis*, has become a commercial source of lumber. Turpentine of this pine has been previously investigated in Russia, France, and New Zealand, with the results as given in Table V.

The oleoresin for the present analysis was obtained from planted trees about twenty years of age and 6 to 8 inches in diameter[1] growing in the Eddy Arboretum of the Institute of Forest Genetics. It was collected from the middle of July to the end of August; the yield was extremely small. The oleoresin solidified in the receptacles into a hard mass.

Upon distillation of about 1700 Gm. of oleoresin, 285 Gm. of turpentine was obtained, which amounted to only 16.8%. The turpentine had the following physical constants:

Density, d_4^{23}	0.8596
Index of refraction, n_D^{23}	1.4727
Specific rotation, $[\alpha]_D$	−5.85°

The oil was fractionated in the usual manner, the results of which are listed in Table VI.

Fraction 3 was tested for α-pinene with amyl nitrite and hydrochloric acid. The crystalline nitrosochloride melted at 106–107° and gave no depression with an authentic sample of α-pinene nitrosochloride.

Fraction 9 was oxidized and nopinic acid, melting point 126–127°, was obtained.

PINUS VIRGINIANA

Virginia pine (*Pinus virginiana*), also called scrub pine, grows in eastern United States from New Jersey and southeastern Virginia to Florida. It is a 2-needle pine, related to *P. radiata*, *P. attenuata*, *P. banksiana*, *P. muricata*, and other pines of the group Insignis. Recently this pine attracted the attention of pathologists who found that a certain fungus (of the genus *Fusarium*) causes an excessive flow of oleoresin (*4*). Experiments have been conducted in inoculating a healthy pine with the fungus in order to increase the flow of gum.

Oleoresin used in the present tests was received from Dr. George H. Hepting, Plant Pathologist of the Bureau of Plant Industry, stationed at the Southeastern Forest Experiment Station, Asheville, N. C. The oleoresin represented a mixed sample obtained from 9 trees treated with the fungus and 2 control (untreated). Upon distillation under reduced pressure, 23% of turpentine was obtained, with the following characteristics:

Density, d_4^{26}	0.8563
Index of refraction, $n_D^{27.5}$	1.4657
Specific rotation, $[\alpha]_D^{25}$	−3.83°

From the physical constants of the fractions (Table VII) it can be concluded that the oil consists mainly of *dl*-α-pinene (90%) with 8% *l*-α-pinene.

The nitrosochloride prepared from fraction 3 melted at 106–107°, and showed no depression of melting point when mixed with α-pinene nitrosochloride obtained from other sources.

TABLE V

Author	Reference	Composition	Physical Constants: Density	Index of Refraction	Specific Rotation
Barraud, Marcelle	"L'état de nos connaissances sur la composition des essences de Térébenthine," *Inst. du Pin Bul.*, No. **58**, 95(March, 1929)	34% α-pinene 62% β-pinene 1.6% dipentene 1.7% tails	0.8700^{15}	1.4712	−11.20°
Arbuzov, B., Abramov, A., and Valitova, F.	"A Study of Composition of Oleo Resin of *Pinus insignis*," *Zhur. Obshch. Khim.*, **2**, 376(1932)	40.6% α-pinene 56.4% β-pinene 0.3% camphene 3% high boiling fraction	$0.8685_4^{17.5}$	$1.4776^{14.5}$	− 3.25°
McCombs, T. H.	"Turpentine from Pines," *New Zealand J. Sci. Technol.*, **12**, 333(1931)	24% α-pinene 75% β-pinene 1% other subst.	0.872–0.874	.....	−12.4°

SUMMARY

Turpentine was obtained under reduced pressure from oleoresin samples of the following species: *Pinus strobus*, *P. cembra*, *P. taeda*, *P. radiata*, and *P. virginiana*.

The oleoresin of *Pinus strobus* consisted of 75 per cent rosin and 25 per cent volatile oil (turpentine). The major constituents of the volatile oil are from *dl*-α-pinene (75 per cent) and *l*-β-pinene (15 per cent). Small amounts of terpene alcohols and ketones (4 per cent) and tricyclic sesquiterpene (0.3 per cent) were found.

The oleoresin of *Pinus cembra* consisted of 78.6 per cent rosin and 21.4 per cent turpentine. The turpentine contained 85 per cent *d*-α-pinene and 9 per cent *l*-β-pinene.

Pinus taeda oleoresin consisted of 85 per cent *d*-α-pinene and 12 per cent *l*-β-pinene. Limonene was not found.

The oleoresin of *Pinus radiata* consisted of 83 per cent rosin and 17 per cent turpentine. The

[1] Tapping of trees was done by Robert Roseman, at that time an employee at the Institute.

TABLE VI.—FRACTIONAL DISTILLATION OF THE VOLATILE OIL FROM *Pinus radiata*

Fraction	Distillation Range Pressure, Mm.	Distillation Range Temperature, ° C.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	740	100–154	1	....	....	.....
2	740	154–155	27	0.8578	1.4690	− 1.73°
3	740	155–157	22	0.8587	1.4693	− 3.84°
4	740	157–158	11	0.8601	1.4718	− 7.57°
5	740	158–159	7	0.8628	1.4743	−11.90°
6	740	159–160	6	0.8659	1.4734	−13.40°
7	740	160–161	2	0.8666	1.4746	−14.47°
8	740	161–162	5	0.8638	1.4746	.....
9	740	162–163	15	0.8660	1.4748	−13°
10	740	163–169	2	....	....	.....
Residue	...	.....	2	....	....	.....

TABLE VII.—FRACTIONAL DISTILLATION OF THE VOLATILE OIL FROM *Pinus Virginiana*

Fraction	Distillation Range Pressure, Mm.	Distillation Range Temperature, ° C.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	740	153–154	41	0.8552	1.4680	−2.36°
2	740	154–155	17	0.8543	1.4703	−5.50°
3	740	155–156	40	0.8546	1.4704	−4.66°
4	Residue	.....	2	....	....	.....

turpentine contained approximately 75 per cent *dl*-α-pinene and 22 per cent *l*-β-pinene.

Pinus virginiana oleoresin consisted of 77 per cent of rosin and 23 per cent of turpentine. The turpentine consists almost entirely of α-pinene (90 per cent of *dl*-α-pinene and 8 per cent of *l*-α-pinene).

Thus composition of turpentines of the five pine species studied is very simple, consisting in four cases chiefly, if not completely, of α- and β-pinenes and in one case of α-pinene alone.

Chemical composition of turpentine of *Pinus cembra* (*sibirica*) from Russia, as reported by Shkateloff, and that of *P. cembra* (a geographically separate tree) from Austria, as found by us, is apparently very similar. On the other hand, composition of *P. strobus* turpentine was found to be different from the morphologically closely related but geographically separated *P. monticola*. While these two species are difficult to distinguish by external characters, their turpentines are different inasmuch as *P. monticola* (from California) contains *n*-undecane and *P. strobus* does not. Analysis of *P. monticola* turpentine from Idaho is in progress.

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Composition of Gum Turpentine of *Pinus lambertiana**

By N. T. MIROV, A. J. HAAGEN-SMIT, and JAMES THURLOW

The composition of gum turpentine obtained from *Pinus lambertiana* has been determined and the results of the study are reported.

SUGAR PINE, or *Pinus lambertiana*, is the most majestic of all pines, reaching a height of 225 feet and a diameter of 10 feet. It grows from southern Oregon to lower California. In California, where it is best developed, it is found in mixture with other conifers at middle elevations of mountain ridges. Botanically it belongs to the subgenus *Haploxylon* or the white pines.

STUDIES

In 1913, turpentine of sugar pine growing at an elevation of 5800 feet was obtained by steam distilla-

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tion and analyzed by Schorger (1) with the following results:

Turpentine in oleoresin	16 4%
Density, d_{15}^{15}, of turpentine	0 8663
Index of refraction, n_D^{15}	1 4728
Specific rotation, $[\alpha]_D^{20}$	+10 42°

The composition of turpentine was reported as follows:

d-α-pinene	70–75%
β-pinene	about 5%
Probably phellandrene	2–3%
Probably aliphatic hydrocarbon	2–3%
A sesquiterpene of an aromodendrene type	

Oleoresin for the present work was obtained by the members of the Institute of Forest Genetics in 1947 in the Sierra Nevada Mountains, at an elevation of about 3500 feet. Twenty trees yielded, in two months, about 10,000 Gm. of honey-like oleoresin that remained liquid after prolonged storage. In some samples rosin acids precipitated reluctantly; in others they did not precipitate at all. Turpentine was obtained by distilling the oleoresin under reduced pressure. Toward the end of the distillation, the temperature inside the flask was 200° and the pressure was 0.02 mm.

The turpentine was distilled at atmospheric pressure until the temperature reached 187° and the distillation was then continued at 38 mm. until the temperature reached 183°. Finally the pressure was reduced to 0.1 mm. and the distillation was discontinued when the temperature reached 250°. The distillation characteristics of the turpentine and the physical constants of the fractions are listed in Table I.

Several high-boiling fractions were analyzed for carbon and hydrogen. The results of this analysis are listed in Table II.

The fractionation data show an accumulation of material at 154–162° and at 166–175° at atmospheric pressure. The presence of α-pinene in the interval 154–156° was shown by the preparation of *dl*-α-pinene nitrosochloride, having a melting point of 112°. No melting point depression was observed when the nitrosochloride was mixed with an authentic pinene nitrosochloride. The oxidation of fraction 164–166° gave nopinic acid, melting point 125°. From the fractionation data and the physical constants of the fraction we may conclude that the distillate contains approximately 65% *l*-α-pinene and 13% *l*-β-pinene.

Judging from its physical constants, fraction 11, boiling point 175–180°, contained a monocyclic

TABLE I.—FRACTIONAL DISTILLATION OF THE VOLATILE OIL FROM *Pinus lambertiana*

Fraction No.	Distillation Range: Pressure, Mm.	Distillation Range: Temperature, ° C.	Distillate, %: Observed	Distillate, %: Cumulative	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	742	148–152	0.4	...	....	1.4581	.....
2	742	152–154	4.6	5.0	0.8536	1.4656	−15.52°
3	742	154–156	29.6	34.6	0.8536	1.4667	−16.56°
4	742	156–158	14.0	48.6	0.8541	1.4691	−17.75°
5	742	158–160	9.4	58.0	0.8554	1.4713	−19.12°
6	742	160–162	5.6	63.6	0.8554	1.4734	−19.24°
7	742	162–164	3.7	67.3	0.8565	1.4743	−19.62°
8	742	164–166	1.4	68.7	0.8577	1.4749	−18.72°
9	742	166–170	3.7	72.4	0.8577	1.4749	−17.30°
10	742	170–175	3.6	76.0	0.8577	1.4750	−16.37°
11	742	175–180	1.6	77.6	0.8536	1.4738	−12.12°
12	38	88–103	0.4	78.0	0.8774	1.4808	+ 0.43°
13	38	103–123	0.6	78.6	0.8995	1.4884	+12.94°
14	38	123–133	2.7	81.3	0.9169	1.4970	+27.84°
15	38	133–141	7.4	88.7	0.9187	1.4983	+32.49°
16	38	141–143	3.0	91.7	0.9187	1.4993	+33.30°
17	38	143–148	3.3	95.0	0.9169	1.4997	+31.36°
18	38	148–158	1.2	96.2	0.9164	1.5005	+25.30°
19	38	158–163	0.6	96.8	....	....	.....
20	38	163–183	0.7	97.5	....	....	.....
21	0.1	130–137	1.1	98.6	0.8937	1.4993	+ 7.35°
22	0.1	137–144	0.6	99.2	0.8995	1.5015	+ 4.38°
23	0.1	144–248	0.4	99.6	....	1.5165	.....
esidue	...	.....	0.4	100.0	....	....	.....

A measure of 5690 Gm. of oleoresin yielded 1045 L. of turpentine (or 18.4%), having the following characteristics:

Density, d_4^{24}	0.8669
Index of refraction, $n_D^{27.5}$	1.4753
Specific rotation, $[\alpha]_D^{27.5}$	−7.46°

Schorger's turpentine was dextrorotatory. In the present tests, 15 turpentine samples, obtained from individual trees, were analyzed. The specific rotation $[\alpha]$ varied from −18.25° to −1.35°. That is, all 15 samples were lævorotatory.

TABLE II.—CARBON-HYDROGEN ANALYSES OF DISTILLATION FRACTIONS OF *Pinus lambertiana* TURPENTINE

Fraction	Temperature Range, ° C.	C, %	H, %	Total
14	123–133 at 38 mm.	86.16	11.59	97.75
15	133–141 at 38 mm.	87.24	11.96	99.20
16	141–143 at 38 mm.	87.63	11.70	99.03
17	143–148 at 38 mm.	87.84	11.81	99.65
20	163–183[a] at 38 mm.	83.39	11.54	94.93
21	130–137 at 0.1 mm.	87.70	11.95	99.65
22	137–144 at 0.1 mm.	87.66	11.97	99.63

[a] Semisolid.

terpene with two double bonds. This fraction did not yield any crystalline derivative with the methods used for the identification of dipentene, terpinene, and carene.

Fraction 12, boiling point 83–88° at 38 mm. or 181–187° at 740 mm., contains a mixture of hydrocarbon- and oxygen-containing derivatives. Terpineol, borneol, as well as other alcohols, were absent as shown by the Tschugaeff and Zerewitinoff (2) method. Saponifications showed the presence of only traces of ester. It is probable that this fraction contains some isomerization and oxidation products of pinene.

Fractions 15–18, boiling between 133 and 158° at 38 mm., contain hydrocarbon as seen from Table II. Attempts at preparing various halogen derivatives from these fractions were unsuccessful. Dehydrogenations were carried out on these fractions according to the method of Melville (3). The absence of any blue color in the dehydrogenated material showed the absence of azulenic sesquiterpenes. A phosphoric acid-petroleum ether partition confirmed this. Picrates were prepared from both the phosphoric acid portion and the petroleum ether portion. The melting points were found to be 114–115°, as compared to Melville's value of 115–116° for cadalene picrate.

The molecular refraction found for fractions 14, 15, 16, 17, and 18 are, respectively, 65.19, 65.24, 65.34, 65.50, and 65.61. For a sesquiterpene with 2 double bonds and 2 rings the molecular refraction, M_D, would be 66.1. These fractions contain, therefore, bicyclic sesquiterpenes of cadalene type structure.

Fraction 20, b. p. 163–183° at 38 mm., contained white crystals which could be separated from the oil on a porous plate. After recrystallization from ethyl alcohol, the crystals melted at 133°. Analysis showed that the crystals were composed of 80.67% of carbon and 11.94% of hydrogen. Calculated for a sesquiterpene alcohol $C_{15}H_{26}O$, the composition would be: carbon 81.08%; hydrogen 11.71%. Molecular weight of the crystalline substance was: found 213; calculated molecular weight for $C_{15}H_{26}O$, 229. Since this alcohol differs from all known sesquiterpene alcohols, the name Lambertol is introduced for this compound.

The fractions distilling at 0.1 mm. from 130–144° consist of hydrocarbons, probably diterpenes.

Saturated hydrocarbons were not present as shown by a treatment of the oil with fuming sulfuric acid.

SUMMARY

The volatile oil of *Pinus lambertiana* contains 65 per cent *l*-α-pinene, 13 per cent *l*-β-pinene, 10 per cent bicyclic sesquiterpene of cadalene type, 2 per cent sesquiterpene alcohol, $C_{15}H_{26}O$, m. p. 133° (Lambertol), and 2 per cent unidentified polyterpenes. It is seen that the results of the present investigation are at variance with the results obtained by Schorger. Although presence of α- and β-pinenes was verified, phellandrene and an aliphatic hydrocarbon were not detected. On the other hand, the sesquiterpene fraction was more fully explored.

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The Decomposition of Sodium Phenobarbital in Solutions of Aliphatic Amines and Alkanolamines*

By MELVIN F. W. DUNKER

The decomposition of aqueous solutions of sodium phenobarbital proceeds at room temperature in the presence of aliphatic amines and alkanolamines to produce the previously reported phenylethylacetylurea. The mixtures had *p*H values ranging between 10.2 and 10.6, whereas the aqueous solution of sodium phenobarbital had a *p*H of 9.2. In some cases crystals of the ureide formed within fifteen minutes after mixing the liquids.

IN CONNECTION with the reaction product of theophylline and phenobarbital, first noted with an aqueous solution containing aminophylline and sodium phenobarbital (1), it was called to the author's attention (2) that an aqueous solution of sodium phenobarbital containing ethylene diamine within a few days deposited white crystals melting at 147–148°. The instability of aqueous solutions of soluble phenobarbital and other barbiturates has been reported in several papers summarized by Husa and Jatul (3) On the basis of the melting point, mixed melting point with the ureide prepared directly from ethylphenylacetic acid, and analytical data, it was evident that the white crystalline solid was ethylphenylacetylurea previously reported as one of the decomposition products of sodium phenobarbital.

* Received Dec. 15, 1948, from the College of Pharmacy, Wayne University, Detroit, Mich.

Stabilized solutions of soluble phenobarbital and other barbiturates are reported in the literature (4–15), in many cases nitrogenous compounds of some type being present, such as amides (6–9), urethane (10, 11), alkanolamines (11), a water-soluble pyrazolone (12), and even diethylamine (13).

The stability of approximately 1.3 molar solutions of sodium phenobarbital in water at room temperature with the 22 amines listed in Table I was investigated. The *p*H values given for the resulting solutions may be considered as approximations since the Coleman *p*H meter was set against a buffer (*p*H 10) but no other correction was applied. The flasks containing *n*-propylamine, di-*n*-butylamine, *n*-amylamine, and 2-aminopentane developed crystals within ten to thirty minutes. The remainder of the solutions developed crystals in two to seven days, the solutions containing the alkanolamines requiring the longer periods. Lack of miscibility did not prevent the formation of crystals as may be noted by observing the behavior of the mixtures containing di-*n*-butylamine, 2-amino-octane, 2-ethyl-*n*-hexylamine, and di-*n*-butylaminoethanol.

EXPERIMENTAL[1]

To a solution of 5.08 Gm. (0.02 mole) sodium phenobarbital in 10 ml. water in a 25- or 50-ml. Erlenmeyer flask was added a mixture of 2 ml. water and 0.02 mole of amine (measured by pipette graduated in hundredths of a ml.). After thorough mixing, the *p*H of each homogeneous solution was immediately measured, using a glass electrode in a Coleman *p*H meter, the instrument being adjusted against a buffer having a *p*H of 10. In three cases (*n*-propyl- and *n*-amylamine and 2-aminopentane) crystals were beginning to form as the solution was being returned to the Erlenmeyer flask from the cup of the *p*H meter. The flasks were stoppered and set aside at room temperature (varying from 70° to 80° F.) for periods up to five weeks. The solution of sodium phenobarbital alone was observed over a period of four months. As crystals accumulated, the first crops were filtered off on Gooch funnels in two to ten days depending upon the rate of formation, the filtrate being returned to the flask. After thorough draining, the solid was washed with a small amount of cold water, the washing being added to the flask. The solid was partially dried by sucking air through it and then put in a desiccator over calcium chloride. After several days in the desiccator, the solids were weighed and the melting points taken. It was difficult to remove all of the amine from the solid in those cases where the mixture was not homogeneous. The residue of water-insoluble amine is no doubt responsible for the larger weights of solid obtained in these cases as well as the low melting points of the crystals obtained, usually in the first crop.

Up to four crops of crystals were filtered off over a period of up to five weeks in exactly the same way, depending on the rate of deposition. Crops of crystals having similar melting points were combined and recrystallized from hot ethanol. The material obtained from the mixtures in flasks 2 to 5, 7, 17 to 21, and 23 just as obtained from the reaction mixture, melted at or very near the melting point of ethylphenylacetylurea. All of these upon one recrystallization from ethyl alcohol melted at 148° ± 0.5° and gave no depression of melting point when mixed

[1] Melting points are uncorrected. The ethylenediamine was kindly supplied by Dr. L. A. Sweet of Parke, Davis & Co., and the remainder of the amines, except the triethanolamine, were supplied through the courtesy of Dr. P. W. Smith of Sharples-Solvents.

TABLE I.—THE FORMATION OF PRECIPITATES IN SOLUTIONS OF SODIUM PHENOBARBITAL AND ALIPHATIC AMINES

Flask No.	Amine	Appearance	*p*H	First Appearance of Crystals	Total Wt. of Solid	Wt. of Solid M. 144–149°
1	.....	Homogeneous	9.2	2 days[a]	0.302	0.275
2	$(H_2N)_2C_2H_4$	Homogeneous	10.4	4 days	1.907	1.907
3	$C_2H_5NH_2$	Homogeneous	10.6	4 days	2.063	2.063
4	$(C_2H_5)_2NH$	Homogeneous	10.7	4 days	1.998	1.998
5	$(C_2H_5)_3N$	Homogeneous	10.6	5 days	1.866	1.866
6	n-$C_3H_7NH_2$	Homogeneous	10.6	10 min.	2.353	1.424
7	i-$C_3H_7NH_2$	Homogeneous	10.6	4 days	2.154	2.154
8	n-$C_4H_9NH_2$	Homogeneous	10.6	4 days	2.840	2.669
9	$(n$-$C_4H_9)_2NH$	2 layers	...	30 min.	2.508	0.431
10	s-$C_4H_9NH_2$	Homogeneous	10.6	4 days	2.912	1.989
11	i-$C_4H_9NH_2$	Homogeneous	10.7	5 days	2.224	1.996
12	n-$C_5H_{11}NH_2$	Homogeneous	10.8	30 min.	3.681	1.684
13	$C_3H_7(CH_3)CHNH_2$	Homogeneous	10.7	2 days	3.105	1.142
14	$(C_2H_5)_2CHCH_2NH_2$	Homogeneous	10.6	5 days	3.641	0.542
15	n-$C_6H_{13}(CH_3)CHNH_2$	2 layers	...	2 days	3.550	[b]
16	n-$C_4H_9(C_2H_5)CHCH_2NH_2$	2 layers	...	2 days	4.090	[b]
17	$(C_2H_5)_2NC_2H_4OH$	Homogeneous	10.4	4 days	1.282	1.282
18	$C_2H_5NHC_2H_4OH$	Homogeneous	10.4	3 days	1.710	1.710
19	$C_2H_5N(C_2H_4OH)_2$	Homogeneous	10.2	7 days	0.666	0.666
20	$CH_3CHOHC_2H_4NH_2$	Homogeneous	10.5	3 days	1.945	1.945
21	$(C_2H_5)_2NC_2H_4NH_2$	Homogeneous	10.6	3 days	2.230	2.230
22	$(n$-$C_4H_9)_2NC_2H_4OH$	2 layers	...	7 days	0.651	0.428
23	$(HOC_2H_4)_3N$	Homogeneous	10.0	7 days	0.453	0.453

[a] Only 3 crystals. After 1 month 0.200 Gm. of solid was filtered off.
[b] In both of these cases, several crystallizations were necessary to get sharp melting crystals. Some phenobarbital was isolated and considerable amounts of phenylethylacetylurea were obtained.

with a sample of ethylphenylacetylurea prepared from ethylphenylacetic acid by way of the acid chloride and recrystallized from ethyl alcohol.

A Kjeldahl determination on a sample of the ureide obtained in the ethyle' 'amine experiment gave the following values:

Theoretical for: $C_{11}H_{14}O_2N_2$, % N 13.59. Found: % N 13.06, 13.50.

The first crop of crystals from flask 6 weighed 0.92 Gm. and melted at 171–174°. Upon recrystallization from ethanol, the material melted at 173–174° and when mixed with U. S. P. phenobarbital, melted at 172–173°. The recrystallization of the solids obtained in flasks 15 and 16 also yielded some phenobarbital. An explanation of this finding is not immediately apparent.

SUMMARY

The decomposition of aqueous solutions of sodium phenobarbital in the presence of 22 aliphatic amines and alkanolamines at room temperature results in the formation of phenylethylacetylurea. The decomposition proceeds more slowly in the solutions of alkanolamines than in the solutions of the aliphatic amines.

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Evaluation of the Antibacterial Efficiency of Dilute Solutions of Free Halogens*,†

By LOUIS GERSHENFELD and BERNARD WITLIN

DILUTE SOLUTIONS of the halogens, in particular, free bromine, free iodine, and chlorine, have been recommended from time to time as general antiseptic washes to be used for wounds and as irrigations.

Due to the physical properties of the free halogens, they cannot be used in solutions of precisely similar constitution, yet the manners in which they are used are essentially comparable. Chlorine, because of high volatility, is customarily used as a hypochlorite, and in solution the Cl^- ion and the OCl^- ion are in such equilibrium as to permit the reaction: $HCl + HOCl \rightleftarrows Cl_2 + H_2O$ to occur. Br_2 and I_2 may be used in solution directly.

The composition of the solutions used in this study are similar to those reported in the literature and are those most likely to be employed in practice. They are a hypochlorite solution; a free bromine solution, a bromine-bromide solution, and an iodine-iodide solution. These formulas are the closest approach to a scheme where the three halogens can be compared on the same basis.

The first controlled experiments in wound healing were conducted by Carrel and Hartmann (1), Carrel (2), and DuNouy (3), who reported that wound healing occurred in a geometric curve. Recently Smelo (4), Anderson (5), and Arey (6) have confirmed the fact that wound healing followed a regular predictable course, and that infection is the most important factor in delaying wound healing.

Prior to the introduction of antiseptic surgery, preparations of free bromine were employed for gangrenous infections (7); and dilute solutions were used for certain types of respiratory infections (7).

Carrel pioneered the irrigation technique for deep infected wounds with Dakin (dilute sodium hypochlorite) solution and later with Dichloramine-T (8–10). Daufresne (11) and others (12, 13) also employed hypochlorous acid.

LaWall and Tice (14) recommended the use of a dilute solution of iodine in isotonic solution of sodium chloride for irrigation purposes.

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Babcock (15, 16) reported on the use of dilute bromine solutions for use as wet compresses and for the irrigation of odorous wounds and fistulas.

The experimental studies on the use of dilute solutions of free halogens, as reported in the literature, have been conducted on each halogen independently; furthermore, different techniques were employed in the investigations. The purpose of this study was to compare the *in vitro* bactericidal efficiencies of dilute solutions of free bromine, free iodine, and chlorine under identical test conditions.

EXPERIMENTAL

Stock solutions of the free halogens were prepared as described in the following paragraphs.

Chlorine.—Solutions of chlorine (as sodium hypochlorite) 1:5000 (0.02%) were prepared by diluting a sodium hypochlorite solution containing 5% available chlorine to the desired concentration with distilled water. The solutions were standardized as to available chlorine content in accordance with the A. O. A. C. procedure (17).

Bromine.—Solutions of free bromine, 1:5000 (0.02%) by weight, were prepared by placing 0.2 Gm. of liquid bromine in a glass-stoppered liter volumetric flask, adding distilled water to 1000 cc., and mixing until solution was effected.

Iodine.—Solutions of free iodine 1:5000 (0.02%) were prepared by diluting 2% Iodine Solution (N. F. VIII) and/or 2% Iodine Tincture (U. S. P. XIII) to 0.02% (1:5000) concentrations with distilled water.

Another series of 0.02% solutions of free bromine, free iodine, and chlorine were prepared as above using isotonic solution of sodium chloride (0.9%) instead of distilled water as the diluent.

(A) Bactericidal Efficiencies of Various Solutions as Determined by a Modified F. D. A. Technique

The bactericidal efficiencies of the solutions of free bromine, free iodine, and chlorine were determined at 24° and 37° by a modified F. D. A. technique (18) employing twenty-four-hour-old cultures of *Staphylococcus aureus, Salmonella (Eberthella) typhosa, Pseudomonas aeruginosa, Escherichia coli*, vegetative forms of *Bacillus mesentericus*, vegetative forms of *Bacillus megatherium*, and vegetative forms of *Bacillus subtilis* as the test organisms. The only modification of the F. D. A. technique was the additional use of shorter time intervals for the first five minutes of the test. Standard F. D. A. loopfuls (18) were transplanted into 10-cc. quantities of sterile F. D. A. broth at one-, two-, five-, ten-, and fifteen-minute intervals. Transplants were incubated for forty-eight hours at 37° and the presence or absence of growth was recorded.

Tests for free halogens were performed at the conclusion of the time-testing period. Free chlorine was detected by the addition of acidified starch-potassium iodide solution and also with orthotolidine. Free bromine was detected by the addition of an excess of potassium iodide to the solution and the liberated iodine detected with starch test solution (U. S. P.) (19). The presence of free iodine was noted by the use of starch test solution (U. S. P.).

Findings.—Five cubic centimeters of 1:5000 (0.02%) chlorine were ineffective within fifteen minutes against 0.5 cc. of twenty-four-hour cultures of the above-mentioned bacteria at 24° and 37°.

Five cubic centimeters of free bromine in the concentrations listed below were effective at 24° within one minute against 0.5 cc. of twenty-four-hour cultures of the following organisms:

S. aureus, 1:6000; *S. (E.) typhosa*, 1:10,000; and *E. coli*, 1:7500; but ineffective within fifteen minutes against the other organisms mentioned above. Five cubic centimeters of 1:5000 (0.02%) free bromine were effective only against *S. aureus* within one minute at 37°, but ineffective within fifteen minutes at 37° against the other above-mentioned organisms.

Five cubic centimeters of the following concentrations of 1:5000 (0.02%) free iodine were effective within one minute at 24° and 37° against 0.5 cc. of twenty-four-hour cultures of the following test organisms:

	24°	37°
S. aureus	1:12,500	1:20,000
S. (E.) typhosa	1: 9,000	1:12,500
E. coli	1:12,500	1:18,000
P. aeruginosa	1:10,000	1:15,000
B. mesentericus (vegetative)	1: 7,500	1:10,000
B. megatherium (vegetative)	1: 7,500	1:10,000

Five cubic centimeters of 1:5000 (0.02%) iodine were ineffective against 0.5 cc. of a twenty-four-hour culture of *B. subtilis* (vegetative) within fifteen minutes at both 24° and 37°.

(B) Bactericidal Efficiencies of Dilute Solutions of Free Bromine, Free Iodine, and Chlorine in the Presence of Citrated Human Plasma

Solutions of free bromine, free iodine, and chlorine were prepared in 1:5000 (0.02%) concentrations in isotonic solution of sodium chloride containing 5% of human citrated plasma. Another series of 1:5000 (0.02%) solutions of the free halogens were prepared as above with isotonic solution of sodium chloride containing 10% of human citrated plasma.

Bactericidal efficiency tests were conducted at both 24° and 37° using the same test bacteria as given under (A).

Findings.—Five cubic centimeters of solutions of 1:5000 (0.02%) chlorine in the presence of 5% and 10% citrated human plasma were ineffective against 0.5 cc. of twenty-four-hour cultures of *S. aureus, S. (E.) typhosa, P. aeruginosa, E. coli, B. mesentericus* (vegetative), *B. megatherium* (vegetative), and *B. subtilis* (vegetative) within fifteen minutes at 24° and at 37°.

Five cubic centimeters of solutions of 1:500[illegible] (0.02%) bromine (*w/v*) were capable of killing 0.[illegible] cc. of twenty-four-hour cultures of *S. aureus, S. (E.) typhosa*, and *E. coli* in the presence of 5% citrated human plasma within one minute at 24°.

Bromine solutions (1:5000) were ineffective against twenty-four-hour cultures of all of the [illegible]teria tested (under A) at 24° in the presence of 10% citrated human plasma and at 37°[1] in the presence of either 5% or 10% citrated human plasma.

Free Iodine.—Five cubic centimeters of [illegible] of free iodine 1:5000 (0.02%) were effective at 37[illegible]

[1] Bromine appears to exert greater bactericidal efficiency [illegible] room temperature (24°) than at body temperature (37[illegible]). This is probably due to the instability of bromine at hi[illegible] temperatures.

gainst 0.5 cc. of *S. aureus*, *S.* (*E.*) *typhosa*, *E. coli* nd *P. aeruginosa* in the presence of 5% citrated human plasma. Half cubic-centimeter quantities of S. (*E.*) *typhosa* and *E. coli* were the only organisms under test killed within one minute at 24° upon exposure to 5 cc. of 1:5000 (0.02%) free iodine in the presence of 5% citrated human plasma. *S.* (*E.*) *typhosa* and *E. coli* were not killed within fifteen minutes at 24° by 1:5000 (0.02%) iodine in the presence of 10% citrated human plasma. *B. mesentericus* (vegetative), *B. megatherium* (vegetative), and *B. subtilis* (vegetative) were not killed by free iodine 1:5000 (0.02%) in the presence of 5% or 10% citrated human plasma at 37° or 24° within fifteen minutes.

(C) Effect of *p*H

Solutions of free bromine, free iodine, and chlorine were freshly prepared in 1:5000 (0.02%) concentrations in McIlvaine's and in Sørensen's buffer solutions at *p*H 2.2, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0.

Five cubic centimeters of the solutions were tested for bactericidal efficiency at 37° against 0.5 cc. of twenty-four-hour cultures of the test organisms (see under A). The buffer solutions at varying *p*H values were likewise tested for bactericidal efficiencies.

Findings.—Fresh solutions of chlorine (1:5000) and fresh solutions of free bromine (1:5000 *w/v*) prepared in McIlvaine's or Sørensen's buffer solutions at *p*H 2.2, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 were not effective against *S. aureus*, *S.* (*E.*) *typhosa*, *P. aeruginosa*, *E. coli*, *B. mesentericus* (vegetative), *B. megatherium* (vegetative), and *B. subtilis* (vegetative) at 37°.

Freshly prepared free iodine solutions (1:5000) at *p*H 2.2, 3.0, 4.0, and 5.0 killed *S. aureus*, *S.* (*E.*) *typhosa*, *E. coli*, *P. aeruginosa* and *B. megatherium* (vegetative) within one minute. *S. aureus* and *S.* (*E.*) *typhosa* were killed within one minute at *p*H 6.0, 7.0, and 8.0 and *E. coli* at *p*H 6.0 and 7.0. *B. mesentericus* (vegetative) was killed within one minute at *p*H 2.2, 3.0, and 4.0. *B. subtilis* was not killed by 0.02% free iodine (1:5000) within the *p*H range of 2.2 to 8.0.

None of the test bacterial cultures used were killed within fifteen minutes by the buffer solutions at *p*H 2.2, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 under conditions of the test.

CONCLUSIONS

I. Bactericidal efficiency tests of dilute solutions (1:5000) of the free halogens prepared either in distilled water or in an isotonic solution of sodium chloride revealed the data presented below.

Chlorine.—Five cubic centimeters of a 1:5000 (0.02%) solution of chlorine were ineffective at both 24° and at 37° against 0.5 cc. of twenty-four-hour cultures of *S. aureus*, *S.* (*E.*) *typhosa*, *P. aeruginosa*, *E. coli*, *B. mesentericus* (vegetative), *B. megatherium* (vegetative), and *B. subtilis* (vegetative).

Free Bromine.—Five cubic centimeters of a 1:5000 solution of free bromine were capable of killing 0.5 cc. of twenty-four-hour cultures of *S. aureus*, *S. typhosa*, and *E. coli* at 24° but ineffective against the remainder of the test organisms (listed under "Chlorine") within fifteen minutes at 24°.

At 37°, 5 cc. of a 1:5000 (0.02%) free bromine solution were capable of killing 0.5 cc. of a twenty-four-hour culture of *S. aureus* within one minute but were ineffective against the other test organisms (listed under "Chlorine") within fifteen minutes.

Free Iodine.—Five cubic centimeters of free iodine solutions 1:5000 (0.02%), prepared by diluting U. S. P. Iodine Tincture or Iodine Solution (N. F.) with distilled water or isotonic solution of sodium chloride, were effective within one minute at both 24° and at 37° against 0.5 cc. of twenty-four-hour cultures of *S. aureus*, *S.* (*E.*) *typhosa*, *E. coli*, *P. aeruginosa*, *B. mesentericus* (vegetative), and *B. megatherium* (vegetative), but were ineffective against *B. subtilis* (vegetative) under the same conditions.

II. Solutions of free bromine, free iodine, and chlorine were prepared in 1:5000 (0.02%) concentrations with distilled water or in isotonic solution of sodium chloride each containing either 5% or 10% of citrated human plasma. The following findings were disclosed.

Chlorine.—Chlorine was ineffective against *S. aureus*, *S.* (*E.*) *typhosa*, *P. aeruginosa*, *E. coli*, *B. mesentericus*, *B. megatherium*, and *B. subtilis* in the presence of 5% and 10% citrated human plasma.

Free Bromine.—Five cubic centimeters of 1:5000 (0.02%) bromine solutions (*w/v*) were capable of killing 0.5 cc. of twenty-four-hour cultures of *S. aureus*, *S.* (*E.*) *typhosa*, and *E. coli* within one minute at 24° in the presence of 5% citrated human plasma, but were ineffective against the same organisms at 37°. Five cubic centimeters of 1:5000 bromine in 5% citrated human plasma were ineffective against 0.5 cc. of *P. aeruginosa*, *B. mesentericus* (vegetative) *B. megatherium* (vegetative), and *B. subtilis* (vegetative) at 24° and at 37°.

Five cubic centimeters of 1:5000 bromine were ineffective at 24° and 37° against all the test organisms in the presence of 10% citrated human plasma.

Free Iodine.—Five cubic centimeters of iodine 1:5000 within one minute killed 0.5 cc. of cultures of *S. aureus*, *S.* (*E.*) *typhosa*, *P. aeruginosa*, and *E. coli* in the presence of 5% citrated human plasma at 37°. Half cubic-centimeter quantities of *S.* (*E.*) *typhosa* and *E. coli* were killed within one minute at 24° when exposed to 5 cc. of 1:5000 iodine in the presence of 5% citrated human plasma, but not in the presence of 10% citrated human plasma. *B. mesentericus* (vegetative), *B. megatherium* (vegetative), and *B. subtilis* (vegetative) were not killed by iodine 1:5000 in the presence of 5% or 10% citrated human plasma at 24° or 37° within fifteen minutes.

III. Solutions of free bromine, free iodine, and chlorine, prepared in 1:5000 concentrations in McIlvaine's and Sørensen's buffer solutions (20) at varying *p*H values (2.2, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) revealed the following: Five cubic-centimeter solutions of chlorine 1:5000 and solutions of free bromine 1:5000 prepared in buffer solutions at *p*H values of 2.2, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 were ineffective at 37° against 0.5 cc. of twenty-four-hour cultures of *S. aureus*, *S.* (*E.*) *typhosa*, *P. aeruginosa*, *E. coli*, *B. mesentericus* (vegetative), *B. megatherium* (vegetative), and *B. subtilis* (vegetative).

Five cubic centimeters of 1:5000 free iodine solutions buffered at *p*H 2.2, 3.0, 4.0, and 5.0 killed *S. aureus*, *S.* (*E.*) *typhosa*, *E. coli*, *P. aeruginosa*, and *B. megatherium* (vegetative) in one minute at 37°. In addition *S. aureus* and *S.* (*E.*) *typhosa* were killed within one minute at *p*H 6.0, 7.0, and 8.0 and *E.*

coli at *p*H 6.0 and 7.0. *B. mesentericus* (vegetative) was killed in one minute at *p*H 2.2, 3.0, and 4.0. *B. subtilis* (vegetative) was not killed by 1:5000 free iodine within the *p*H range of 2.2 to 8.0.

SUMMARY

Iodine solutions in concentrations of 1:5000 (0.02 per cent) in either distilled water or isotonic solution of sodium chloride or either of the two diluents each containing 5 per cent citrated human plasma, displayed more effective antibacterial activity against several test bacteria either at 24° or 37° as compared to solutions of chlorine (1:5000) or free bromine (1:5000 *w/v*). The test bacteria used were broth cultures of: *Staphylococcus aureus, Salmonella* (*Eberthella*) *typhosa, Pseudomonas aeruginosa, Escherichia coli,* vegetative forms of *Bacillus megatherium,* vegetative forms of *Bacillus mesentericus,* and vegetative forms of *Bacillus subtilis.*

Weak iodine solutions (1:5000) displayed findings as above in *p*H ranges from 2.2 to 8.0. Identical concentrations of free bromine and chlorine under similar tests were ineffective.

Five-cubic-centimeter quantities of McIlvaine's and Sørensen's buffer solutions (as controls) at *p*H 2.2, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 did not kill 0.5-cc. quantities of the test bacterial cultures within fifteen minutes at 24° and 37°.

Solutions of free iodine 1:5000 (0.02 per cent) for use as an antiseptic wash or for irrigations were readily prepared by diluting 1 cc. of 2 per cent Iodine Solution (N. F. VIII) and/or 2 per cent Iodine Tincture (U. S. P. XIII) to 100 cc. with distilled water or isotonic solution of sodium chloride.

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Book Reviews

An Introduction to Materia Medica and Pharmacology. By ELSIE E. KRUG and HUGH ALISTER MCGUIGAN, 5th ed. C. V. Mosby Co., St. Louis, 1948. 558 pp. 13.5 x 21.5 cm. Price $4.

Pharmacology and Therapeutics in Nursing. By MARION S. DOOLEY and JOSEPHINE RAPPAPORT. McGraw-Hill Book Co., New York, 1948. xi + 444 pp. 15 x 23.5 cm. Price $3.75.

To determine how much pharmacology to teach a nurse is not easy. Such a decision may depend upon whether she is considered to be a person in the professional or subprofessional category. Modern nursing schools with their added emphasis upon the teaching of academic subjects would seem to suggest that she is considered professional.

If the nurse is to have status as a professionally trained person, she should have a thorough groundwork in the principles of drug action and she must know the main and side actions of drugs to a far greater extent than either of these books suggests. With the increasing number of new drugs becoming available every month and with most hospitals participating in the clinical testing of new drugs, more responsibility is placed in the nurse's hands than ever before. She does not need the detailed knowledge of the medical student, but it would seem that she needs more information than either of these books gives.

The reviewer believes that the Dooley-Rappaport text is restricted to too few drugs. For example, only the official barbiturates are considered, despite the fact that the nurse will be confronted with many commercial barbiturates. It is important for her to be familiar with the names, doses, and the type or category to which these barbiturates belong.

The Krug-McGuigan text, on the other hand, is a little less discriminating in the selection of drugs, but is not quite so careful in the accuracy of its facts. For example, on Page 149, nicotinamide is said to be the same as coramine (which is diethylnicotinamide) and is said to have a nicotine-like action.

:s structural similarity to nicotine is emphasized espite the fact that the compound has been reamed niacinamide in order to de-emphasize this :ructural similarity.

There are a number of groups—nurses, veterinaans, pharmacists and dentists, to name a few—who re interested in pharmacology from a different iewpoint than that of the physician. It is to be oped that some day the "ideal" book will be availble for these groups.

urface-Active Agents—Their Chemistry and Technology. By ANTHONY M. SCHWARTZ and JAMES W. PERRY. Interscience Publishers, New York, 1949. xi + 579 pp. 15 x 23 cm. Price $10.

During the past decade there has been a greatly ncreased interest in surface-active agents and surace chemistry. Although there have been many ooks written in this field, most of them have been vritten from the "practical" viewpoint and have een incomplete in background information. This ook by Schwartz and Perry combines the theoreti:al and the practical in a most effective manner.

In an introductory chapter the authors summarize general information concerning surface chemistry very briefly. This is followed by a section on processes for synthesizing and manufacturing surfaceictive agents. In this section the surface-active agents are divided into chemical types and each type s discussed from the viewpoint of the laboratory synthesis and commercial production. This sec:ion, as is the entire book, is well documented. Especially helpful are the many references to the patent literature.

The second division of the book concerns the physical chemistry of surface-active agents. Here one finds discussions on surface and interfacial relationships of pure liquids, surface properties of solutions, the bulk properties of surface-active solutions, the gross effects and technical evaluation of such agents, and the relationship of surface activity to chemical constitution discussed in an admirable manner. Although the facts and theories are concisely expressed, nothing of importance has been omitted.

The last portion of the book is given over to the practical aspects of surface chemistry. Of particular interest to pharmacists are chapters on "cosmetics and personal use" and "pharmaceutical, germicidal, fungicidal, and disinfectant uses."

A full author and subject index complete the book.

This is the book on surface-active agents that pharmaceutical chemists have been looking for. The only thing that it lacks is a list of the characteristics of trade-named products and their manufacturers.

Industrial Hygiene and Toxicology. Volume I. Edited by FRANK A. PATTY. Interscience Publishers, Inc., New York, 1948. xxvii + 531 pp. 16 x 25 cm. Price $10.

Industrial hygiene, having awakened widespread interest since the 1920's, is a relatively new field in the United States. In Europe, it dates back farther, having interested Paracelsus, among others. Despite its youth, it has made rapid strides, however, both in the assembling of large masses of data and in broadening its scope. It is fitting that this excellent reference, by many authorities, should become available at this time.

Much of the earlier work in the field of industrial hygiene revolved around toxic gases and fumes, industrial dusts, and machinery guards to prevent maiming. Modern industrial hygiene has gone far beyond this treatment phase into prophylaxis. The industrial hygienist now seeks to inculcate positive health into the worker so as to promote efficiency and well-being. For this reason it is not surprising to find the chapters by Josef Brozek and W. N. Witheridge on the effects of personal factors and environmental factors on competence and fatigue.

Frank A. Patty, the editor, has written the introductory chapter on "retrospect and prospect," as well as a chapter on the survey and personnel and chapters on the mode of entry and action of toxic materials, sampling and analysis of atmospheric contaminants, and respirators and respiratory protective devices.

Pharmacists will be particularly attracted to the chapter by Dr. Louis Schwarz on occupational dermatosis, for in this section will be found a discussion on the use of protective ointments. Pharmacists in industrial areas frequently can cooperate with the industrial hygienists in the development of such ointments and creams.

Other important chapters found in the first volume of this two-volume reference deal with the physiological effects of abnormal atmospheric pressure, radiant energy and radium, ventilation, the visible marks of occupation and occupational diseases, fire and explosion hazards of combustible gases and vapors and combustible dusts, and the role of dust in the causation of occupational disease.

To those with the responsibility of the health of relatively large numbers of people, this book will be of interest. It is helpful, not only for the factual information, but for the investigative approach consistently maintained.

We are looking forward to Volume II.

Newer Synthetic Analgesics. Consulting Editor, M. L. TAINTER. New York Annals of the New York Academy of Sciences. New York, 1948. 174 pp. 15 x 24 cm. Price $2.75.

The New York Academy of Sciences is famous for the high quality and practicality of its symposia. During 1948 one of the outstanding symposia was that on newer synthetic analgesics, the subject of this issue of the *Annals*.

This symposium was particularly timely because several outstanding drugs have appeared in this field recently and new developments in testing have also come upon the scene.

The symposium opened with an excellent historical review on pain by Dr. M. L. Tainter. The chemistry of natural and synthetic analgesics was then reviewed by Dr. Lyndon F. Small. Dr. Carl C. Pfeiffer and co-workers next reviewed experimental methods for studying analgesia. Dr. Lloyd C. Miller gave a critique of analgetic testing methods.

The more general papers on chemistry and pharmacology were followed by papers dealing with

specific drugs such as Methadone, Metopon, Dermerol, and related compounds. Such eminent pharmacologists as Nathan B. Eddy, F. F. Yonkman, and K. K. Chen presented the papers in this group.

The next group of papers dealt with the addiction problem and were presented by M. H. Seevers, Harris Isbell, and Robert C. Batterman.

Dr. E. E. Nelson and Mr. H. J. Anslinger, respectively, discussed the relations of the Food and Drug Administration and the Bureau of Narcotics to new analgesic agents.

The final papers dealt with clinical application of analgesics and were presented by Raymond N. Bieter, E. A. Rovenstine, Bert B. Hershenson, R. W. Houde, and others.

All papers presented and bound together in this issue of the *Annals* were of exceptionally high quality. An amazing amount of information is covered on these pages. The reader is brought up to date in this important field in an effective and interesting manner. Almost uniformly the authors have pointed out gaps in our knowledge, thus stimulating the research worker.

The Academy is to be congratulated upon its choice of subject matter and authors for this excellent symposium.

Pharmacologic Principles of Medical Practice. By JOHN C. KRANTZ, JR., and C. JELLEFF CARR. Williams & Wilkins Co., Baltimore, Md., 1949. xv + 980 pp. 15 x 23 cm. Price $10.

Probably no supporting science to the field of medicine has made greater progress during the past decade than pharmacology. Not only have a wealth of new drugs entered the picture, but the drugs have been of such a nature that whole concepts of drug action have been changed or, at least, have been modified. Because of these advances, a new textbook has been badly needed and Drs. Krantz and Carr have met that need very well.

The authors have consistently emphasized their theme of pharmacologic *principles* in medical *practice.* Never is basic principle sacrificed for obscure detail and yet rarely absent is the detail necessary for intelligent practice. In addition, the facts are marshaled in a readable and teachable manner. The human interest approach is used throughout and given emphasis by an excellent collection of portraits of the leaders in the field—Magendie, Abel, Fleming, Spalding (too few pharmacologists appreciate his contribution), A. J. Clark, Waksman, Domagk, Wells, Sertürner, Chen, Hunt, McCollum, and others.

The drugs are grouped according to physiologic processes into antiinfective drugs, the response of skin and mucous membranes to drugs, the response of the central nervous system, the autonomic nervous system, the heart and circulation, and the reproductive system to drugs, and the effect of pharmacologic agents on metabolism.

The appendix carries an interesting section on the discovery and evaluation of new drugs as well as a series of typical prescriptions.

The introduction contains the usual introductory information—scope, history, nature and source of drugs, drug control, the prescription, general principles of drug action, and much more. Many phases of this section are difficult to handle brief and fair manner. The authors have essentially factual although their interpreta the position and scope of the National Fo is not quite accurate. It is not true that which are prescribed at least 10,000 times a are admitted to the National Formulary matically, although extent of use is an imp factor in determining scope. The authors that the National Formulary is in a seco position relative to drug standards, but the rev maintains that, while its scope is different from of the United States Pharmacopœia, the book less important. From the viewpoint of p health, it would seem to be just as important drugs which are widely used should be standar even though they may not bear the stam approval of the Pharmacopœia.

Farmaseuttisia Ja Kemiallisia Synonymeja. Y. W. JALANDER. Helsingfors, 1948. 570 pp. + 47-p. supplement. 12 x 19 cm.

This book comprises a list of about *21,000* onyms intended for the use of the Scandina countries, more particularly Finland. A of comparative acid strengths of the various tries throughout the world is included also.

Although the reviewer believes this book to inestimable value to the pharmacists for whom written, it may be of limited interest only to A can pharmacists who are interested in foreign and foreign prescriptions.

Basic Facts of Health Education for Members of Medical, Pharmaceutical and Nursing Pro sions. The Pharmaceutical Press, 17 Bloomsb Square, London, W.C.I., England. vi + 193 13.5 x 21 cm. Price 7*s.* 6*d.*

Basic Facts of Health Education is a collectio essays selected from British Ministry of He Bulletins and which appeared in the *Pharmaceu Journal* (London) during 1944–1947. These Bu tins were supplied to British pharmacists to them informed of developments about which might be consulted by the general public and to sist them in the dissemination of useful informati

From the scope of the topics covered it is ev that the British, too, are becoming increasin aware of the significance of the pharmacist source of public health information. Some of topics covered are communicable diseases measles, chicken pox, and scarlet fever, all which are discussed in separate essays in an au tative and clear manner.

Some other interesting topics presented are n natal care: its scope and extent; the work of a cal officer of health; the importance of pure supplies; food poisoning; cancer—some facts treatment and early symptoms; dysmenorrh notes on constipation remedies; eradication of in wartime; mass miniature radiography; co and cough mixtures; stored blood and plas ries; and many other cogent subjects.

Many American pharmacists will wish to copy of this handbook at their prescripti since it contains much useful information on he education.